## REMARKS/ARGUMENTS

This paper request entry under 37 CFR 1.116 and 27 CFR 1.195 of the attached evidence consisting of (1) Transmittal, (2) Supplemental Communication and Press Release, and (3) Copy of Communication to the Examiner filed May 20, 2003 (52pp.), (4) Postcard showing receipt of (1), (2) and (3) by the PTO on July 22, 2003.

The Press Release dated June 14, 2003 describes the results of a phase II clinical trials on patients with LADA treated with GAD. This clinical trial was referred to in the Baekkeskov declaration filed May 20, 2003. However, the results of the trial were not available when the declaration was filed. The Press Release describing the results was filed promptly after the results became available and before the issuance of a final rejection (which issued July 29, 2003). The Press Release and accompanying papers were filed by hand delivery and a postcard stamped by Technical Center 1600/2900 on July 22, 2003 was received. Applicants were unaware that these papers may not have been entered into the record until receiving a telephone call from the Examiner on this subject on or around November 15, 2004.

The 52-page Communication first filed May 20, 2003 was refiled on July 22, 2004 in view of the Examiner apparently not having received it. However, it appears from the PTO electronic file wrapper that the PTO did receive and enter at least part of the Communication. The Communication consists of a Baekkeskov declaration, curriculum vitae of Dr. Baekkeskov and copies of references cited in the declaration. It appears that the Baekkeskov declaration and curriculum vitae were entered into the record when the original communication of May 20, 2003 was filed. However, the attached references were apparently not entered either on the original filing of May 22 or the refiling of July 22. In any event, a complete copy of the 52-page communication filed both May 20, 2003 and July 22, 2003 is provided here.

The standard for entry of a declaration or exhibits with an appeal brief is provided by 37 CFR 1.195, which states:

> Affidavits, declarations, or exhibits submitted after the case has been appealed will not be admitted without a showing of good and sufficient reason why they were not presented earlier.

Here, the papers for which entry is requested were submitted earlier before final rejection. In particular, the Press Release was submitted very soon after it was released (released June 14, 2003, submitted July 22, 2003). The 52-page communication was submitted on May 20, and again on July 22, 2003. However, the Press Release was apparently not entered into the record at all, and the 52-page communication was entered only in part due to misplacement in the PTO. That the papers were misplaced by the PTO provides good and sufficient reason why they were not officially entered into the record earlier and why they should now be entered.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,

J. Liebeschuetz

Joe Liebeschuetz
Reg. No. 37,505

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
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Attachments
JOL:jol
60360694 v1

**FILING ACKNOWLEDGMENT**
**VIA HAND DELIVERY**

Please stamp the date of receipt of the following document and return this card to us:



File No.: 2307AA-031220US JOL/klc
Inventors: Steinunn Baekkeskov *et al.*
Appln. No.: 08/838,486 Date Filed: April 7, 1997
Title: IMPROVED METHODS FOR THE DIAGNOSIS AND TREATMENT OF DIABETES

Enclosed:
(1) Transmittal Form (1 pg);
(2) Supplemental Communication to Examiner and copy of referenced press release (4 pages); and
(3) Copy of Communication to the Examiner filed 5/20/03 (52 pages)

**FILING ACKNOWLEDGMENT**
**VIA HAND DELIVERY**

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File No.: 2307AA-031220US JOL/klc
Inventors: Steinunn Baekkeskov *et al.*
Appln. No.: 08/838,486 Date Filed: April 7, 1997
Title: IMPROVED METHODS FOR THE DIAGNOSIS AND TREATMENT OF DIABETES

Enclosed:
(1) Transmittal Form (1 pg);
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(3) Copy of Communication to the Examiner filed 5/20/03 (52 pages)

PA 3319707 v1

PTO/SB/21 (05-03)
Approved for use through 04/30/2003. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE


# TRANSMITTAL FORM

*(to be used for all correspondence after initial filing)*

| | |
|---|---|
| Application Number | 08/838,486 |
| Filing Date | April 7, 1997 |
| First Named Inventor | Steinunn Baekkeskov *et al.* |
| Art Unit | 1644 |
| Examiner Name | G. Ewoldt |
| Attorney Docket Number | 2307AA-031220US |
| Total Number of Pages in This Submission | 57 |

**ENCLOSURES** ***(Check all that apply)***

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
  - ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s)
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group **(Appeal Notice, Brief, Reply Brief)**
- ☒ Other Enclosure(s) *(please identify below)*:

Supplemental Communication to the Examiner and copy of referenced press release (4 pages)

Copy of Communication to the Examiner filed 5/20/03 (52 pages)

Return Postcard

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|---|---|
| Remarks | The Commissioner is authorized to charge any additional fees to Deposit Account 20-1430. |

**SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT**

| | |
|---|---|
| Firm or Individual | Townsend and Townsend and Crew LLP<br>Joe Liebeschuetz Reg. No. 37,505 |
| Signature | J. Liebeschuetz |
| Date | July 21, 2003 |

**CERTIFICATE OF TRANSMISSION/MAILING**

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| | | | |
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PATENT
Attorney Docket No.: 2307AA-031220US
Client Ref. No.: 90-160-5

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

Steinunn Baekkeskov *et al.*

Application No.: 08/838,486

Filed: April 7, 1997

For: IMPROVED METHODS FOR THE DIAGNOSIS AND TREATMENT OF DIABETES

Examiner: G. Ewoldt

Art Unit: 1644

SUPPLEMENTAL COMMUNICATION TO THE EXAMINER

Tech Center 1600
Crystal Mall I
7th Floor Receptionist
1911 South Clark Place
Arlington, VA 22202

Sir:

Applicants attach an additional copy of a submission mailed May 20, 2003. It appears from the PAIR records that the original has not been entered into the case. The submission of May 20, 2003 contains the executed declaration of Dr. Baekkeskov which is to be referred to in subsequent proceedings in this case

The Declaration of Dr. Baekkeskov filed May 20, 2003 referenced to a Phase II clinical trial to test the efficiency of GAD in preventing patients from becoming insulin dependent (a symptom of insulin-dependent diabetes).

Applicants now provide a press release summarizing the results of the trial. In brief, the trial showed administration of GAD is safe and provided statistically significant evidence of efficacy.

Respectfully submitted,

J. Liebeschuetz

Joe Liebeschuetz
Reg. No. 37,505

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PA 3319698 v1

DIAMYD
MEDICAL

Press Release

Stockholm, June 14, 2003

# DIAMYD REPORTS SUCCESSFUL CLINICAL PHASE II TRIAL WITH DIABETES VACCINE

*Diamyd Medical AB, publicly traded on the Stockholm Stock Exchange in Sweden (O-list), today reported a positive outcome from a phase II trial with its GAD-based diabetes vaccine Diamyd™. The results may lead to a new treatment to prevent type 1 diabetes.*

The presentation of the results took place at the American Diabetes Association (ADA) convention in New Orleans, by Dr. Åke Lernmark, University of Washington, Seattle. "My opinion is that this phase II study has been tremendously successful", says Åke Lernmark "Not only is it now shown that the Diamyd™ vaccine can be safely administrated in a wide range of doses, but a clear and significant positive effect (P=0.01) of the vaccine was found at one of the dose levels six months from first vaccination. It is also important to note that the trial was conducted to the highest standards which ads further weight to its results."

"We could not have hoped for better results. The Diamyd™ vaccine is safe and we have an effective dose to go on with", says Anders Essen-Möller, President and CEO of Diamyd Medical.

In type 1 diabetes, the immune system mistakenly destroys the insulin-producing cells in the pancreas in an autoimmune attack. Over time, this attack leads to a lack of insulin, the hormone that controls blood sugar levels. People with type 1 diabetes must inject insulin daily.

In type 2 diabetes, patients normally continue to produce their own insulin but are less sensitive to it. Therefore these patients may be treated with tablets to increase their sensitivity to insulin. A large group (about 10%) of the type 2 diabetes patients have antibodies to GAD. These patients are called LADA and suffer from a similar autoimmune attack as the type 1 diabetes patients, which leads to the need for insulin injections.

Diamyd Medical conducted the phase II clinical trial by vaccinating patients with recently diagnosed LADA. The GAD-vaccine successfully improved these patients' C-peptide levels and therefore their ability to make insulin over a six-month period, compared with patients who received a placebo.

"The study shows that the vaccine is safe and that it is possible to inhibit the autoimmune attack on the cells that make insulin, thereby slowing the progression of the disease," said Essen-Moller.

The vaccine to prevent type 1 diabetes arose from experiments with diabetes prone-mice that were protected from developing the disease by injecting GAD-protein. "It's tremendously satisfying to see our work at UCLA go from the lab to a clinical application with the potential to help so many people" said Daniel Kaufman, Ph.D., Professor, UCLA Department of Molecular and Medical Pharmacology, whose research team was first to develop and test a GAD-vaccine in diabetes-prone mice.

Diamyd Medical's phase II trial was conducted on 47 diabetes patients with the GAD-based vaccine Diamyd™ at the UMAS hospital in Malmoe and St.Gorans Hospital in Stockholm, Sweden. The patients were randomly divided into four groups with 12 patients in each group. Each patient received one first injection of Diamyd™ followed by at least

one boost injection four weeks after. Nine patients in every group received active drug whereas three received placebo. The groups received different doses of the vaccine ranging from 4 to 500 micrograms per dose. All patients visited the hospitals 10 times during this six-month study, and detailed clinical, immunological as well as neurological investigations showed no safety concerns at the administered dose levels.

The study results show that the diabetes vaccine significantly improves the serum C-peptide levels both at fasting (P=0.01) and after meals (P=0.02) at one of the doses.

"Since the vaccine seem effective when given to people with an advanced disease, we are hopeful that it will be highly effective when given at earlier stages of the disease process—we now know that type 1 diabetes takes years to develop and that we can detect people who are at early stages of the disease process by testing for GAD autoantibodies in their blood" said Essen-Moller

"We will now continue to analyze the results from this study" says Essen-Möller. The future for the Diamyd™ diabetes vaccine is promising".

Diamyd Medical is identifying and developing therapeutic candidates through phase II. The Company's intention is thereafter to seek co-operation with established pharmaceutical companies for further development. Diamyd Medical is pursuing various GAD-based development projects of which the GAD-based diabetes vaccine Diamyd™ is the most advanced at this time. Diamyd Medical has licensed exclusive and worldwide intellectual rights for therapeutic use of GAD from the Universities of California in Los Angeles and University of Florida in Gainesville, Florida.

The first application for Diamyd™ is older patients with adult onset diabetes with GAD antibodies since this patient group progress to full insulin dependence within a few years. The market for this application may be in the area of one billion US dollars per year. Future studies will address whether the vaccine can also prevent the development of type 1 diabetes in young people that have not yet developed the disease. With the availability of a potential therapeutic, the pre-diagnostic tests for who is at risk for developing the disease (based on the detection of antibodies to GAD and other islet proteins), becomes quite valuable. Diamyd has an extensive array of pre-diagnostic kits for detecting autoantibodies to these proteins. Additional possible applications of the vaccine are to prevent recurrent autoimmune diabetes after transplantation of islet cells and stem cell therapy.

**About Diamyd Medical:**

Diamyd Medical's business idea is to identify and develop pharmaceutical projects up to and including Phase II. At present Diamyd Medical is running a number of GAD-based development projects and has the licensed rights for this from universities in the US.

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**For further information, please contact:**

Johannes Falk, Diamyd Medical AB (publ),
Phone: +46 8-661 00 26, +46 8-661 12 25,
fax: +46 8-661 63 68, or via e-mail: info@diamyd.com

*No guarantee is given or implied for the accuracy of any statements on present, historical or future results.*

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DIAMYD
MEDICAL

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

On May 20, 2003

TOWNSEND and TOWNSEND and CREW LLP

By: Paula Fault Hurley

PATENT
Attorney Docket No.: 2307AA-031220US
Client Ref. No.: 90-160-5

COPY

# IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Steinunn Baekkeskov *et al.*

Application No.: 08/838,486

Filed: April 7, 1997

For: IMPROVED METHODS FOR THE DIAGNOSIS AND TREATMENT OF DIABETES

Examiner: G. Ewoldt

Art Unit: 1644

COMMUNICATION TO THE EXAMINER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Attached is an original executed declaration of Dr. Steinnun Baekkeskov, a copy of her curriculum vitae and certain articles cited in the declaration.

This original declaration should replace that filed with the response of May 5, 2003. It has been discovered that the fax signature sheet of this declaration was inadvertently attached to a draft rather than the completed version of the declaration. The draft was not executed by Dr.Baekkeskov and should be ignored. To avoid confusion between the draft and the executed declaration, applicants have attached a cover page

titled "Original executed declaration of Dr. Steinnun Baekkeskov." This is to be referred to in subsequent proceedings.

References cited in Dr. Baekkeskov's declaration, for which copies are not provided here, are believed to be already of record. However, copies can be provided if the Examiner is not able to find the cited references.

Respectfully submitted,

J Liebeschuetz

Joe Liebeschuetz
Reg. No. 37,505

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PA 3305683 v1

Original Executed Declaration
of Dr. Steinnun Baekkeskov

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

On May 20 2003

TOWNSEND and ~~TOWNSEND~~ and CREW LLP

By: (Paula Faulk Hurley) Paula Faulk Hurley

PATENT
Attorney Docket No.: 2307AA-031220US
Client Ref. No.: 90-160-5

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Steinunn Baekkeskov *et al.*

Application No.: 08/838,486

Filed: April 7, 1997

For: IMPROVED METHODS FOR THE DIAGNOSIS AND TREATMENT OF DIABETES

Examiner: G. Ewoldt

Art Unit: 1644

DECLARATION OF STEINUNN BAEKKESKOV

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Steinunn Baekkeskov, state as follows:

(1) I am Professor of Medicine and Microbiology/Immunology, and Horan Markarian Chair of Diabetes at the University of California, an assignee of the above-captioned application. A copy of my curriculum vitae is attached as Exhibit A. I have actively conducted research in diabetes for over twenty years. I regularly read the scientific literature, particularly that relating to diabetes, attend scientific meetings, and am conversant with the view of many colleagues.

 

(2) I have reviewed the above-captioned application of which I am a co-inventor and have followed the prosecution history thereof. I understand that the priority date of the application is September 7, 1990.

(3) The application is in large part premised on the discovery that glutamic acid decarboxylase (GAD) is a component of a pancreatic beta cell 64 kDa antigen that is a major autoantigen in insulin dependent diabetes mellitus (IDDM) (also known as type 1 diabetes). The application discloses administering GAD to a patient to inhibit or prevent IDDM. Administration of GAD induces tolerance to the 64 kDa autoantigen, thereby inhibiting or preventing further destruction of beta pancreatic cells, and the clinical symptoms of IDDM that eventually result from this destruction.

(4) When an antigen is administered to a subject, it can induce either a tolerogenic or an immune response depending on the regime with which it is administered. The application teaches that care should be taken not to potentiate an immune response that would exasperate ß cell destruction. Based on my knowledge of the scientific literature, general principles for achieving a tolerogenic response rather than a harmful immunogenic response were within the state of the art as of September 1990. For example, standard immunology textbooks available at the priority date of the invention discuss how either low or high dosages of antigen favor a tolerogenic response, whereas intermediate dosages favor an immunogenic response (Benjamini & Leskowitz, Immunology: A Short Course (Liss, 1988) at p. 255-256; Golub, The Cellular Basis of the Immune Response (2nd ed. Sinauer, 1981) at page 291). These textbooks also discuss how the use of unaggregated antigen favors a tolerogenic response. Induction of antigen specific tolerance had been used successfully in numerous studies to suppress or prevent autoimmune disease in animal models ((Cremer et al., Collagen induced arthritis in rats: antigen-specific suppression of arthritis and immunity by intravenously injected native type II collagen. J. Immunol. 131, 2995-3000 (1983); Scherer et al., Control of cellular and humoral immune responses by peptides containing

T cell epitopes. Cold Spring Harbor Symp. Quant. Biol. 54, 497-504, 1989; Nagler-Anderson et al., Suppression of type II collagen induced arthritis by intragastric administration of soluble type II collagen. Proc. Natl. Acad. Sci. USA 83, 7443-7446 (1986); Higgins and Weiner. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic proteins and its fragments. J. Immunol. 140, 440-445 (1988)). Given this guidance as to how to generate a tolerogenic response to ameliorate autoimmune disease, I believe scientists in the IDDM field would be able to use the knowledge of an identity of a target autoantigen to devise conditions to obtain a tolerogenic response to prevent or delay disease as of September 1990.

(5) This expectation has been confirmed by numerous reports in the scientific literature in which administration of GAD has been shown to induce tolerance in NOD mice and prevent IDDM (see *e.g.*, Tisch *et al.*, *Nature* 366, 71-75 (1993); Kaufman, *Nature* 366, 69-71 (1993), Tian *et al.*, *Nature Medicine* 12, 1348 (1996), Peterson *et al.*, *Diabetes* 44, 1478 (1994), and Pleau *et al.*, *J. Immunol. Immunopath.* 76, 90-95 (1995)). Several different parenteral routes of administration have successfully been used (see Harrison, *Molecular Medicine* 1, 722-727 (1994)).

(6) The NOD mouse is a good model of the major type of IDDM in which human patients develop autoantibodes and T cells to GAD, because NOD mice also develop autoantibodies and T cells to GAD (see Tisch *et al.*, *Nature* 366, 72-75 (1993) at *e.g.*, p. 21, column 1, first paragraph). The NOD mouse is a genetic strain of mouse that spontaneously develops autoimmunity to GAD, and subsequently symptoms of IDDM, in a manner similar to development of IDDM in humans. Positive results in the NOD mouse have been used as evidence to support human clinical trials of a number of drugs to treat IDDM. For example, human clinical trial of humanized OKT3 to treat IDDM is underway following a showing that such an antibody reversed hyperglycemia in NOD mice (see attached summary of the trial and Herold et al., New Engl. J. Med. 346, 1692-1698 (2002)). Similarly, a human clinical trial of alpha interferon is underway following

a showing that ingestion of alpha interferon prevents diabetes in a NOD mouse (see attached summary of trial). Most importantly, the results using GAD to induce tolerance and prevent diabetes in the NOD mouse have been used as evidence to support human clinical trials of a GAD vaccine to treat human type II diabetic patients (non-insulin dependent). These patients are treated with oral medication, but have autoantibodies to GAD, demonstrating that they are experiencing autoimmune destruction of ß cells and are therefore likely to become insulin dependent. The vaccine has been shown to be safe. The results of phase II of the clinical trials, which may provide an indication of the efficacy of the vaccine in preventing patients from becoming insulin dependent, will be announced at the American Diabetes Association annual meeting in New Orleans, June 13-17, 2003 (see attached summaries of trial).

(7) By contrast, the BB rat is not such a close model of IDDM in humans or other organisms that develop antibodies to GAD. The BB rat bears a genotype that results in spontaneous development of lymphocytopenia and clinical symptoms similar to those of IDDM. However, lymphocytopenia is not found in human IDDM. Furthermore, unlike the NOD mouse, and unlike most humans, the BB rat does not develop autoimmunity to GAD (see Petersen *et al.*, Autoimmunity 25, 129-138 (1997) at p. 134, col. 1). Because the BB rat does not develop autoantibodies to GAD, there is no reason to expect that therapeutic intervention with GAD would have any effect in the BB rat. Therefore, lack of such an effect in the BB rat, cannot be extrapolated to humans or other animals in which autoantibodies to GAD are present.

(8) In my opinion, the above evidence shows that a tolerogenic response has been obtained to GAD in mouse model of IDDM that is protective for IDDM and is predictive of similar response in humans. In my opinion, the evidence further shows this response was obtainable based on the teaching of the specification and common knowledge in the field as of September 1990.

(9) I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

St Baekkeskov

Steinunn Baekkeskov

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 650-326-2400
Fax: 415-576-0300
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PA 3303202 v1

Exhibit A

# STEINUNN BAEKKESKOV

## Curriculum Vitae

**Affiliation** Professor of Medicine and Microbiology/Immunology
Horan Markarian Chair of Diabetes

**Address:** Hormone Research Institute/Diabetes Center, Box 0534
University of California, San Francisco
San Francisco, CA 94143-0534

## EDUCATION

1976: Candidatus Scientarum, (M. Sc./Ph.D.degree) in Biochemistry from the University of Copenhagen, Denmark.

1984: Licentiata scientarum, (Ph.D. degree) in Immunology from the University of Copenhagen, Denmark

## PROFESSIONAL AND RESEARCH EXPERIENCE

1973-1975: Thesis student, Department of Chemistry, The Carlsberg Laboratory, Copenhagen. Isolation characterization and chemical modification of enzymes from *Saccharomyces cerevisae*. Thesis: Characterization and chemical modification of glucose-6-phosphate dehydrogenase from Brewers yeast.

1976 Lecturer in Biochemistry, Department of Biochemistry, University of Copenhagen Medical School

1977-1979: Postdoctoral Fellow, Department of Biochemistry, International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya. Isolation and characterization of membrane proteins and lipids of African trypanosomes.

1980-1982: Postdoctoral Fellow, Hagedorn Research Laboratory, Gentofte, Denmark. Research area: Immunology/cell and molecular biology of the pancreatic β-cell. The role of autoimmunity in the pathogenesis of insulin-dependent diabetes.

1982-1986: Staff Scientist, Hagedorn Research Laboratory.

1986-1989: Senior Staff Scientist, Hagedorn Research Laboratory (permanent position).
1987-1989: Member of a panel of 6 Senior Staff Scientist that formed the Directory Board of the Hagedorn Research Laboratory.

1989-1992: Assistant Professor, Department of Medicine, Department of Microbiology/Immunology, University of California, San Francisco

1992-1998 Associate Professor Department of Medicine, Department of Microbiology/Immunology, University of California San Francisco.

1998-present Professor of Medicine and Microbiology/Immunology, University of California San Francisco

1990-1992: Member of UCSF Graduate Program in Endocrinology
1992-date: Member of UCSF Graduate Program in Molecular Medicine in PIBS
1992-date: Member of UCSF Biomedical Sciences Graduate Program
1993-date: Member of UCSF Graduate Program in Immunology in PIBS
1994-date: Member of UCSF Graduate Program in Cell Biology in PIBS

## AWARDS AND HONORS

| | |
|---|---|
| 1970-1973 | P. Wulff's Foundation Scholarship |
| 1973-1975: | Carlsberg Foundation Research Student Fellowship Award |
| 1982-1984: | Juvenile Diabetes Foundation Fellowship Award |
| 1984-1987: | Juvenile Diabetes Foundation Career Development Award. |
| 1991-1993 | NIH-Shannon Award |
| 1997-current | Horan Markarian Chair of Diabetes |

## PUBLICATIONS

### Original Articles in Reviewed Journals:

1. Rovis, L. and **Baekkeskov, S.** Subcellular fractionation of *Trypanosoma brucei*. Isolation and characterization of plasma membranes. Parasitology **80**, 507-524 (1980).

2. **Baekkeskov, S.**, Kanatsuna, T., Klareskog, L., Nielsen, D.A., Peterson, P.A., Rubenstein, A.H., Steiner, D.F., and Lernmark, A. Expression of major histocompatibility antigens on pancreatic islet cells. Proc. Natl. Acad. Sci. USA **78**, 6456-6460 (1981).

3. Steffes, M.W., Nielsen, 0., Dyrberg, T., **Baekkeskov, S.**, Scott, J., and Lernmark, A. Islet transplantation in mice differing in the I and S subregions of the H-2 complex. Transplantation **31**, 476-479 (1981).

4. **Baekkeskov, S.**, Nielsen, J.H., Marner, B., Bilde, T., Ludvigsson, J., and Lernmark, A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate specific human pancreatic islet cell proteins. Nature **298**, 167-169 (1982).

5. Dyrberg, T., **Baekkeskov, S.**, and Lernmark, A. Specific pancreatic β-cell surface antigens recognized by a xenogenic antiserum. J. Cell Biol. **94**, 472-477 (1982).

6. Dyrberg, T., Nakhooda, A.F., **Baekkeskov, S.**, Lernmark, A., Poussier, P., and Marliss, E.B. Islet cell surface antibodies and lymphocyte antibodies in the spontaneously diabetic "BB" Wistar rat. Diabetes **31**, 278-181 (1982).

7. Kanatsuna, T., **Baekkeskov, S.**, Lernmark, A., and Ludvigsson, J. Immunoglobulin from insulin-dependent diabetic children inhibits glucose-induced insulin disease. Diabetes **32**, 520- 524 (1983).

8. **Baekkeskov, S.**, and Lernmark, A. Glucose stimulates the biosynthesis of a human pancreatic islet cell protein, detected by an antiserum against the human erythrocyte glucose transporter. FEBS Letters **157**, 331-335 (1983).

9. **Baekkeskov, S.**, Dyrberg, T., and Lernmark, A. Autoantibodies against an Mr 64K islet cell protein precede the onset of insulin-dependent diabetes in the BB-rat. Science **224**, 1348-1350 (1984).

10. **Baekkeskov, S.** and Lernmark, A. A β-cell glycoprotein of Mr 40,000 is the major rat islet cell immunogen following xenogenic immunization. Diabetologia **27**, 70-73 (1984).

11. Gerling, I., **Baekkeskov, S.**, and Lernmark, A. Islet cell and 64K autoantibodies are associated with plasma IgG in newly diagnosed insulin-dependent diabetic children. J. Immunol. **137**, 3782-3785 (1986).

12. **Baekkeskov, S.**, Landin, M., Kristensen, J.K., Srikanta, S., Bruining, G.J., Mandrup-Poulsen, T., de Beaufort, C., Soeldner, J.S., Eisenbarth, G., Lindgren, F., Sundquist, G., and Lernmark, A. Antibodies to a Mr 64,000 human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J. Clin. Invest. **79**, 926-934 (1987).

13. Efrat, S., **Baekkeskov, S.**, Lane, D., and Hanahan, D. Coordinate expression of SV40 large T and p53 proteins in β-cells of transgenic mice harboring hybrid insulin-large T antigen genes. Embo J. 6, 2699-2704 (1987).

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DIAMYD MEDICAL

Press Release
Stockholm, April 11, 2003

# Diamyd Medical to present Phase II results with diabetes vaccine in June

*Diamyd Medical (O-list) is developing a GAD vaccine for insulin-dependent diabetes. The company plans to present results from its Phase II trial with the vaccine during the American Diabetes Association Congress in New Orleans, June 13-17, 2003. The study's main aims are, in the first place, to investigate the safety of the vaccine and in the second place to obtain an indication of the vaccine's efficacy. It is Diamyd Medical's ambition, if the Phase II trial is successful, to seek co-operation with an established pharmaceutical company for further comercialization of the vaccine and in doing so to achieve a positive cash flow.*

Diamyd Medical has been conducting a Phase II study since May 2001 on 48 orally treated diabetes patients who have antibodies to GAD. The GAD antibodies indicate that an autoimmune process is underway, that eventually will destroy the patient's insulin-producing cells by which time patients will be dependent on daily insulin injections. The Diamyd vaccine is intended to prevent this development so that patients continue to produce their own insulin. This Phase II study, the results of which are expected to be available in June, is aimed in the first place to investigate if the vaccine is safe to administer as well as to provide an indication of the vaccine's effect at various dose levels. The study is double-blind and placebo controlled, that is nobody knows who has received the active vaccine or the placebo.
"We are working to be able to break the code in June and present the results of the study during the American diabetes conference, which this year is being held in New Orleans June 13-17," says Anders Essen-Möller. "This event is usually attended by some 8,000 doctors and scientists and we hope to reach out with our results to them."

About Diamyd Medical:
Diamyd Medical's business idea is to identify and develop pharmaceutical projects up to and including Phase II. At present Diamyd Medical is running a number of GAD-based development projects and has the licensed rights for this from universities in the US. The Company's project that has come farthest is a vaccine for insulin-dependent diabetes.
The projects will then be sold or licensed to major pharmaceutical companies for further commercialization. The development and marketing of related diagnostic tests and substances takes place in parallel to promote contact with researchers and prepare the market for the impending pharmaceuticals.

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DIAMYD MEDICAL

Press Release
Stockholm, April 28, 2003

# Major potential for Diamyd's diabetes vaccine

*Diamyd Medical (O-list) reports that the last patient samples have been taken today in the ongoing six month's Phase II study with the GAD-based diabetes vaccine, Diamyd™. Diamyd Medical is planning to present the results from the study at the American Diabetes Association scientific congress in the US between June 13 and 17. The first application of the vaccine is seen to be preventing diabetes patients being treated with insulin in tablet form from becoming dependent on injections. Further applications are expected to be the prevention of insulin dependency in children and young people who run the risk of developing the illness and increasing the survival capability of insulin producing cells after transplantation.*

The first diabetes patient was injected with the Diamyd diabetes vaccine in May 2001. Since then, a total of 47 patients have been included in Diamyd Medical's clinical Phase II study that has been carried out in both Malmö and Stockholm. Each patient has visited the hospital ten times during a six-month period so that samples could be taken. Extensive analysis of the samples have been carried out by experts in the US, the UK and Sweden. Apart from the safety aspects of the vaccine, both metabolic and immunological parameters are thoroughly monitored aimed at obtaining an indication of the vaccines function and efficacy. "Today another milestone has been passed with the last four patient samples being taken in the six-month study," says CEO Anders Essen-Möller. "We look forward with great interest to the results that are expected to be published in June at the American Diabetes Association conference in New Orleans."

The first category for the vaccine is seen to be those diabetes patients with antibodies against GAD who are being treated with tablets. The annual market for this patient category is estimated to be SEK 5-10 billion. There are similar markets if the vaccine can be developed to prevent insulin-dependent diabetes in children and young people.

About Diamyd Medical:
Diamyd Medical's business idea is to identify and develop pharmaceutical projects up to and including Phase II. At present Diamyd Medical is running a number of GAD-based development projects and has the licensed rights for this from universities in the US.

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## NEW CLINICAL TRIAL IN NEWLY DIAGNOSED TYPE 1 DIABETES HOUSTON, TEXAS

THE UNIVERSITY OF TEXAS DIABETES RESEARCH GROUP NEWSLETTER presents new information on studies of oral (ingested) type I interferon. The Endocrinology Divisions in both Internal Medicine and Pediatrics are now recruiting newly diagnosed type 1 diabetes patients in a phase II randomized, double-blind, parallel-design clinical trial to determine whether ingested (oral) human recombinant IFN-a will prolong the 'honeymoon' period. We have demonstrated that ingested IFN-a prevents type 1 diabetes in the NOD mouse. Ingested IFN-a also prolongs the 'honeymoon' period in newly diagnosed type 1 diabetics in phase I open label clinical trial recently completed here at UT-Houston. The natural history of type 1 diabetes is unique for a phase frequently referred as the "honeymoon", a period in which the insulin need becomes minimal and glycemic control improves. The b cell partially recovers. However, as with all honeymoons, they end and the patient becomes completely insulin-deficient. The general consensus of the international diabetes community is to test potential preventive therapies for type 1 diabetes in newly diagnosed patients. Prolongation of the honeymoon as the reversal of the disease is considered a positive result.

Entry criteria include male or female type 1 diabetes patients requiring insulin within one month of diagnosis between the ages of 3-25 without concurrent diseases. Eighty eligible patients will be randomized into one of two treatment arms - the active treatment arm will ingest 30,000 units IFN-a daily and the non-active treatment arm will ingest placebo (saline) for one year.

Prior to enrollment into the study (within 1 month of diagnosis), patients will be evaluated in the UT University Clinical Research Center at Hermann Hospital with a complete medical exam and routine blood tests. Patients

will be seen monthly for the first three months, and every three months thereafter. Primary outcome measures will be a 30% increase in C-peptide levels released after Sustacal stimulation at 3, 6, 9, and 12 months after entry. If successful, this will lead to a larger and longer phase III trial of prevention of type 1 diabetes in high risk patients.

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We appreciate your help in referring patients to our Diabetes Research Group. Your efforts allow patients the opportunity to be involved in cutting edge clinical trials. There is no charge to your patients. Patients will continue to be followed by their private endocrinologist for optimization of glycemic control during the course of the study. This trial will require trips to Houston at entry and at months 1, 2, 3, 6, 9, and 12 for testing.

If you have or know of patients that might wish to participate in this clinical trial outlined above, please call any of the numbers below.

Staley A. Brod, MD Principal Investigator - 713 500-7046 or 713 500-7050, Fax:713-500-7041 (PI)
Phil Orlander, MD Adult Endocrinology - Co- Principal Investigator 713-500-6646
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ORIGINAL ARTICLE

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# Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus

*Kevan C. Herold, M.D., William Hagopian, M.D., Ph.D., Julie A. Auger, B.A., Ena Poumian-Ruiz, B.S., Lesley Taylor, B.A., David Donaldson, M.D., Stephen E. Gitelman, M.D., David M. Harlan, M.D., Danlin Xu, Ph.D., Robert A. Zivin, Ph.D., and Jeffrey A. Bluestone, Ph.D.*

## ABSTRACT

*Background* Type 1 diabetes mellitus is a chronic autoimmune disease caused by the pathogenic action of T lymphocytes on insulin-producing beta cells. Previous clinical studies have shown that continuous immune suppression temporarily slows the loss of insulin production. Preclinical studies suggested that a monoclonal antibody against CD3 could reverse hyperglycemia at presentation and induce tolerance to recurrent disease.

*Methods* We studied the effects of a nonactivating humanized monoclonal antibody against CD3 — hOKT3γ1(Ala-Ala) — on the loss of insulin production in patients with type 1 diabetes mellitus. Within 6 weeks after diagnosis, 24 patients were randomly assigned to receive either a single 14-day course of treatment with the monoclonal antibody or no antibody and were studied during the first year of disease.

*Results* Treatment with the monoclonal antibody maintained or improved insulin production after one year in 9 of the 12 patients in the treatment group, whereas only 2 of the 12 controls had a sustained response (P=0.01). The treatment effect on insulin responses lasted for at least 12 months after diagnosis. Glycosylated hemoglobin levels and insulin doses

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were also reduced in the monoclonal-antibody group. No severe side effects occurred, and the most common side effects were fever, rash, and anemia. Clinical responses were associated with a change in the ratio of CD4+ T cells to CD8+ T cells 30 and 90 days after treatment.

*Conclusions* Treatment with hOKT3γ1(Ala-Ala) mitigates the deterioration in insulin production and improves metabolic control during the first year of type 1 diabetes mellitus in the majority of patients. The mechanism of action of the anti-CD3 monoclonal antibody may involve direct effects on pathogenic T cells, the induction of populations of regulatory cells, or both.

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- Tang, Q., Smith, J. A., Szot, G. L., Zhou, P., Alegre, M.-L., Henriksen, K. J., Thompson, C. B., Bluestone, J. A. (2003). CD28/B7 Regulation of Anti-CD3-Mediated Immunosuppression In Vivo. *J Immunol* 170: 1510-1516 [Abstract] [Full Text]
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# Clinical Trial Research Summary

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**A Phase II, multiple dose treatment of Type 1 diabetes with hOKT3γ-1(Ala-Ala**

**Principal Investigator:** Kevan Herold, Columbia University

Abstract | Investigators | News | Background | Resources

## Abstract

**Objective:** The objective of this proposal is to study the immunologic effects of humani non-binding anti-CD3 mAb on immune responses associated with Type 1 diabetes (T1DI develop this therapy to prevent the immune destruction leading to beta cell loss.

**Basis/Rationale:** Studies of the natural history of T1DM indicate that 100% of individu the disease still make detectable insulin even after the first year of diabetes but lose tht completely over the next 5 years. Retention of the ability to produce any insulin endoge results in improved clinical control of the disease, and therefore, reduced secondary complications. In the NOD mouse, anti-CD3 mAb reversed hyperglycemia after presentz hyperglycemia, induced long lasting protection from disease in the absence of continuou treatment, and prevented recurrent diabetes in recipients of islet allografts. Pre-clinical FcR non-binding anti-CD3 mAb suggest that the mAb selectively anergizes activated Th: possibly by delivering an altered TCR signal. Th1 cells are thought to be involved in T1D are most prevalent in the islet at the late stages of the disease, thus suggesting the bas efficacy of anti-CD3 mAb even after presentation. We have been conducting a Phase I/I this agent in patients with new onset T1DM. The drug has been well tolerated and does the toxicities of OKT3. Our studies have suggested a dosing regimen appropriate for Phε trials and mechansms that may account for the mAb effect.

**Significance:** The trial formally tests the hypothesis, that in man, T1DM is mediated by lymphocytes. This drug may be of valuefor treatment of T1DM and prevention of its recu islet allografts. Relevance of Immune Tolerance: In mouse studies, the drug induces tol T1DM. This study will test the same in man, and develop a protocol that will maintain to islet cells.

**Clinical Protocol Summary:** In this Phase II protocol, the mAb will be administered or 1, 2, or 3 occasions during the first 1 1/2 years of disease. This protocol differs from the Phase I, single treatment protocol in that repeated administration of the mAb is utilized the effects of the mAb in a manner analogous to repeated administration of a vaccine. C responses of the treated groups (n=24 in each) will be compared to untreated patients.

**Mechanistic Studies:** The planned mechanistic studies, included in this proposal are sp questions pertaining to the immunologic effects in diabetes, and will determine how the works and how to best utilize it for treatment. The studies test three mechanisms to acc the actions of the drug. First, that pathogenic T cells are deleted from the repertoire by treatment. Second, that the drug anergizes specific populations of T cells, most likely Th cells including those cells responsible for islet antigen recognition. Third, that subpopula cells activated by mAb (i.e. CD69+ or CD25+) represent a regultory population that ma the function and/or effects of autoimmune effector cells. In addition to these studies, sa and clinical information from this trial should facilitate studies using tolerance assays of

## Participating Investigators

- *Kevan Herold,* Naomie Berrie Diabetes Center, Columbia University

- *Jeffrey A. Bluestone,* THe Diabetes Center at UCSF
- *Michael H. Dosch,* University of Toronto, Canada
- *Peter Gottlieb,* Barbara Davis Diabetes Center
- *Carla Greenbaum,* Virginia Mason Center
- *William Hagopian,* Pacific Northwest Research Institute
- *David Harlan,* National Institutes of Health
- *Andrew Muir,* University of Florida, Gainesville
- *Gerry Nepom,* Virginia Mason Center
- *Jerry Palmer,* University of Washington

## News & Recent Developments

- Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus - *NEJM* [go → ]
- Anti-CD3 Monoclonal Antibody Slows New-Onset Type 1 Diabetes - *Medscape* [go → ]

## Background Articles

- Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibod *Diabetes* [go → ]
- CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice - *J Immu* → ]
- Anti-CD3 Antibody Induces Long-Term Remission of Overt Autoimmunity in Nonobes Diabetic Mice - *PNAS* [go → ]
- OKT3γ(ala-ala) delivers partial TCR signal- -*J Immunol* [go → ]

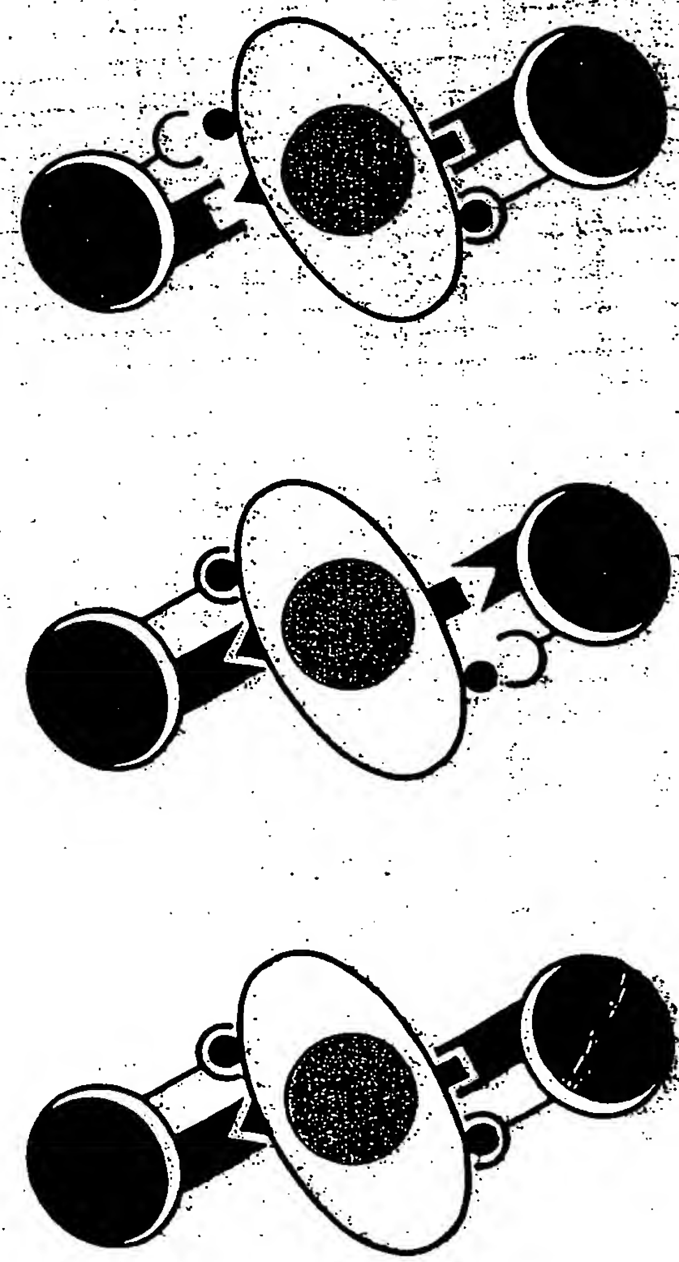
## Resources & Interesting Links

- Tolerogenic strategies to halt or prevent type 1 diabetes Nat Immunol [go → ]

Edward S. Golub

# THE CELLULAR BASIS OF THE IMMUNE RESPONSE

SECOND EDITION



THE COVER

While it is basically true that "one cannot tell a book by its cover," the cover sometimes tells a great deal about the book. The cover logo for the first edition of *The Cellular Basis of the Immune Response* summarized the main theme of the book and the status of cellular immunology in 1977. Helper and effector cells, each reactive to different portions of the antigen molecule, were shown interacting to bring about an immune response.

By 1981 the situation has become more complex and the cover logo of the present edition reflects these changes. The emphasis now is on the T-cell reacting with the macrophage through two sets of receptors. One set of receptors is for antigen and the other is directed against self-MHC antigens. This reflects the central role of the MHC in immune reactions and the need for autoreactivity against MHC.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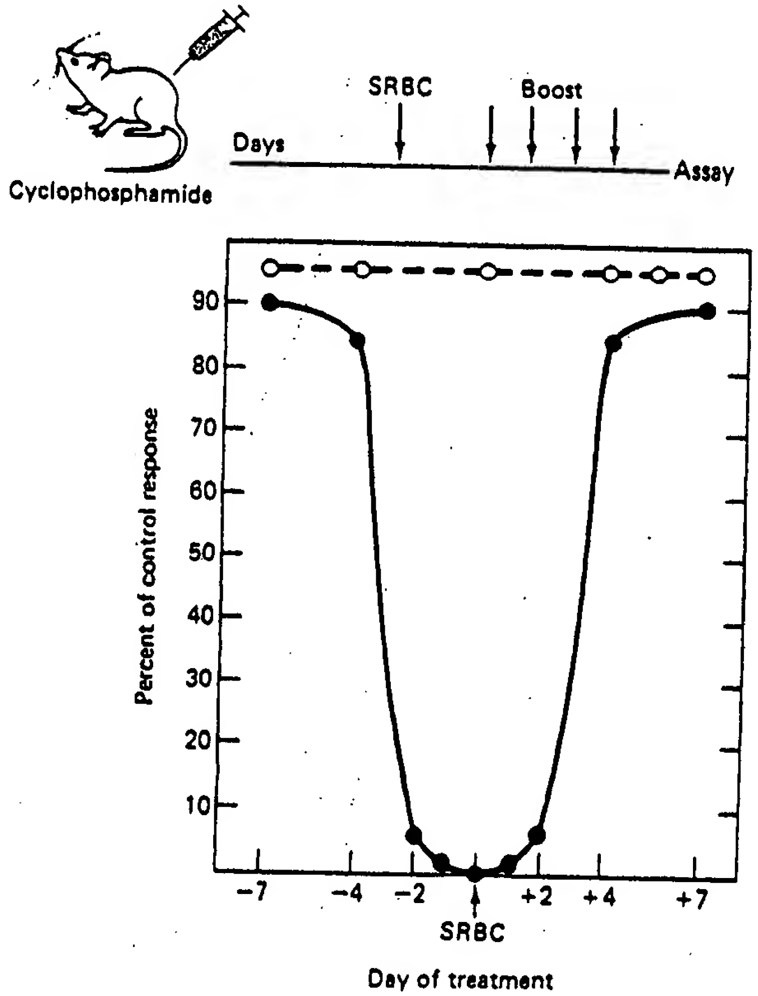
For Jonathan and Mark . . .
who make it all worthwhile




: were treated with cyclophosphamide either before or after ıving SRBC as tolerogen. SRBC were injected several times, and SRBC assayed on day 35. Anti-SRBC response was diminished, ·esponse to non-cross-reacting antigen was normal. [From Aisen- (1967). *J. Exp. Med.* 125, 833.]

e, treatment with the drug 7 or 14 days before SRBC injection ıo effect on subsequent SRBC responses. However, treatment for rrow time span of 3 days before or 3 days after SRBC treatment ted in loss of ability to produce anti-SRBC while the ability to nd to an unrelated antigen was unimpaired. This shows that a of specific tolerance was established to SRBC when the mimetic drug was used at the time that antigen was introduced. ar results have been obtained with BSA in the rabbit using not ·adiomimetic drugs but nonlethal doses of X-irradiation.

*Antigen Form*

The form in which the antigen is injected can also be important in inducing immunity or tolerance. If the antigen is in a form to which the animal cannot respond by producing antibody or a cell-mediated response, a state of tolerance is often established. We have already seen that the aggregate-free form of the antigen induces tolerance. Another example of this is seen when mice are injected with the hapten DNP on a carrier which is nonimmunogenic such as poly-D-GL. This is a random polymer of D-glutamic acid and D-lysine. The L-polymer of poly-GL is immunogenic, but the D-polymer is not. When mice pretreated with the DNP-D-GL polymer are challenged with DNP on the immunogenic carrier KLH, they fail to produce antibody to DNP but make normal amounts of antibody to an unrelated antigen. Thus the DNP-D-GL acts as a tolerogen.

*Antigen Concentration*

Another means of inducing tolerance is to treat animals with *extremes of antigen concentration*. In a phenomenon known as *immune paralysis* discovered by Felton in 1934, mice are treated with either 100 or 10μg pneumococcal polysaccharide (abbreviated S III). The mice which receive the 10μg S III are protected when challenged with virulent pneumococcus organisms, but all those pretreated with 100μg die of the disease. In this case a low dose immunized the mice, but a high dose did not. It was thought that the high dose of antigen "paralysed" the immune system. We now think of this as a form of tolerance in which there is failure of lymphocytes to respond to antigen.

A rather surprising observation was made when *extremely low doses* of protein antigen were used to induce tolerance. In the heroic experiment of Mitchison shown in Figure 4 groups of mice were injected with varying doses of soluble BSA three times a week for up to 16 weeks. In this way the effect of dose and time could be determined. The animals were then challenged with the immunogenic form of BSA, and anti-BSA antibody titers were determined. When the data was plotted, it was found that high doses of tolerogen induced tolerance (as expected) but that very low doses also induced tolerance. This phenomenon is called LOW ZONE TOLERANCE and has now been observed with several antigens. Treatment with middle doses of BSA primed the mice.

*Antibody-Induced Tolerance*

Tolerance can also be induced by administrating *antibody*.

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# COLLAGEN-INDUCED ARTHRITIS IN RATS: ANTIGEN-SPECIFIC SUPPRESSION OF ARTHRITIS AND IMMUNITY BY INTRAVENOUSLY INJECTED NATIVE TYPE II COLLAGEN[1]

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**Collagen-induced arthritis (CIA) developed in 70 to 90% of rats immunized with heterologous type II collagen. CIA was reduced to 0 to 18% when rats were injected i.v., i.e., pretreated, with 1 mg of soluble native type II collagen before immunization. Concomitant with the suppression of CIA were significant suppression of IgM, IgG, and delayed-type hypersensitivity (DTH) responses to type II collagen. Suppression of CIA and immunity to collagen was antigen-specific, related to dose and route of administration, and occurred only when 1 mg of collagen was injected i.v. either 32, 7, or 4 days before, or 7 days after immunization. Once CIA was established, however, neither arthritis nor immunity could be suppressed.**

**To determine if adjuvant-induced arthritis (AIA), like CIA, could be suppressed by i.v. pretreatment with type II collagen, rats were given 1 mg of type II collagen or PBS i.v. before injection with mycobacteria and oil. AIA was not suppressed, and arthritis appeared in both groups at a similar incidence and severity. Sera from 26 rats with severe AIA that was collected between days 14 and 35 after injection were assayed for IgG to homologous rat type II collagen and were found to be negative.**

**These findings further support the hypothesis that CIA in rats is mediated by immunity to type II collagen and also suggest that CIA and AIA have different primary pathogenic mechanisms.**

Collagen-induced arthritis (CIA)[3] is an experimental model of inflammatory polyarthritis that can be induced in 70 to 90% of susceptible rats (1–18) or mice (19–21) by sensitizing them with heterologous native type II collagen. In addition to arthritis, approximately 14% of rats also develop inflammatory auricular chondritis that histologically resemble the lesions found in relapsing polychondritis (8–10).

Although the exact pathogenic mechanism(s) remain to be defined, substantial evidence exists linking CIA with the immune response to type II collagen. Investigators from this laboratory (1–4) and others (5–7) have shown that sera from rats with CIA contain significantly higher titers of antibody to type II collagen than sera from sensitized rats that failed to develop disease. Furthermore, we have shown a temporal relation between the onset of CIA and the presence of circulating IgM and IgG immunoglobulins and lymphocytes reactive with native type II collagen (4). Additional evidence supporting an immune-mediated pathogenesis of CIA includes the adoptive transfer of arthritis to naive recipients (11), abrogation of disease by depleting serum complement (C) with cobra venom (12), modulation of CIA with immunostimulatory and immunosuppressive agents (13), suppression of CIA by i.v. injection of collagen-coated spleen cells (14), and most recently the passive transfer of CIA to naive recipients by using specific IgG prepared from sera of rats with acute arthritis (15, 16).

Reported here are studies showing that a single injection of soluble type II collagen, given i.v., before or shortly after intradermal (i.d.) challenge with an arthritogenic preparation of type II collagen, was highly effective and specific in suppressing CIA and immunity to type II collagen. In contrast, an i.v. injection of type II collagen had no effect on the incidence or severity of AIA. These findings further support the hypothesis that CIA in rats is mediated by immunity to native type II collagen and also suggest that CIA and AIA have different primary pathogenic mechanisms.

## MATERIALS AND METHODS

*Animals.* Outbred female Wistar rats (Charles River Breeding Laboratories, Inc., Wilmington, MA or Harlan Sprague-Dawley, Indianapolis, IN) weighing between 100 and 125 gm were used for these studies. Rats from both suppliers developed CIA at the same incidence and produced virtually identical IgG responses to type II collagen. Rats were housed in groups of five in wire-bottomed metal cages and fed standard laboratory chow and water *ad libitum.*

*Collagen preparation.* Native bovine (BII), chick (CII), and rat (RII) type II collagens were solubilized by limited pepsin digestion of fetal bovine cartilage, chick sterna, and rat chondrosarcoma tumor, respectively; and were purified as described (3). Native bovine type I collagen (BI) was solubilized by pepsin digestion of fetal bovine skin (1). Purity of collagen was determined by amino acid analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and uronic acid analysis.

*Protocol to modify CIA by i.v. injection of solubilized type II collagen.* To determine whether the incidence, severity, or duration of CIA could be modified by injection of soluble type II collagen without incomplete Freund's adjuvant (IFA), rats were injected i.v. with a solution of type II collagen in phosphate-buffered saline (PBS) pH 7.2. This procedure will herein be referred to as pretreatment. To determine the effect of pretreatment on the course of CIA, rats were immunized with BII. Immunization will be referred to as challenge. The dose of collagen given i.v. and time of pretreatment relative to challenge were varied depending on the design of the experiment.

*Preparation of soluble antigens for i.v. injection.* Solutions of collagen for i.v. injection were prepared as follows. Native CII, BII, or BI collagens were dissolved overnight at 4°C in 0.1 M acetic acid (1.2 mg/ml), dialyzed in PBS, and centrifuged at 100,000 × G for 30 min to remove any insoluble collagen.

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[1] This work was conducted under program-directed research funds from the Veterans Administration and supported in part by Research Grant AM-16506 from the National Institutes of Health, the Arthritis Foundation, UTCHS New Faculty Award 00073, and James H. Wood Foundation. Portions of this material were presented at the 45th annual meeting of the American Rheumatism Association, June 3, 1981, Boston, MA and published in Arthritis Rheum. 24:559 (abstr.).
[2] Dr. Cremer is a Postdoctoral Fellow of the Arthritis Foundation. Requests for reprints should be addressed to him at the Veterans Administration Medical Center, Research Service (151), 1030 Jefferson Avenue, Memphis, TN 38104.
[3] Abbreviations used in this paper: AIA, adjuvant-induced arthritis; BI, native bovine type I collagen; BII, native bovine type II collagen; CII, native chick type II collagen; CIA, collagen-induced arthritis; CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; i.d., intradermal; MAI, maximum arthritis index; OVA, ovalbumin; RII, native rat type II collagen; s.c., subcutaneous.

The concentration of collagen in the supernatant was determined by hydroxyproline content and was adjusted to 1 mg/ml before i.v. injection. One group of rats was injected i.v. with 1 mg of 5× recrystalized ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) that was dissolved directly into PBS (1 mg/ml). Intravenous injections were made via the lateral tail vein.

*Immunization protocols.* CIA was induced by immunizing rats with native BII that had been dissolved overnight at 4°C in 0.1 M acetic acid (4 mg/ml) and emulsified with an equal volume of IFA (Difco Laboratories, Detroit, MI). Rats were injected i.d. twice with 200 $\mu$g of emulsified BII. The first injection was made in a hind metatarsal footpad; the second, 7 days later, into the proximal one-third of the tail. In one study, rats were injected first with BII emulsified in complete Freund's adjuvant (CFA) (Difco Laboratories) and then reinjected with BII/IFA 7 days later. Immunization with BI or OVA in IFA was performed in an identical manner as BII.

Adjuvant arthritis was induced by injecting rats i.d. at the base of the tail with 250 $\mu$g of mycobacteria suspended in 0.05 ml of heavy mineral oil (E. R. Squibb and Sons, Princeton, NJ). Mycobacteria strains C.D.T. and P.N. were obtained from the Ministries of Agriculture, Fisheries and Food, Weybridge, Surrey, England, and were ground in an agate mortis and pestle with oil shortly before injection.

*Determination of severity of arthritis.* Rats were examined daily between days 10 and 28 for the presence and severity of disease. Afterward they were examined 2 or 3 times per wk for an additional 2 to 3 mo. Any rat developing CIA within the 4-mo duration of the study was included in calculations of incidence, onset, and severity of disease.

Severity of arthritis was determined subjectively by grading each limb on a scale of 0 to 4 as described (4). The maximum arthritis index (MAI) was calculated for each rat as the sum of the greatest score recorded for each limb. Thus, a score of 0 represents the absence of arthritis, and scores of 1 and 16 the mildest and worst disease, respectively. The MAI per group was calculated by the formula: number of arthritic rats × the mean MAI ÷ by number of rats in the group.

*Collection of blood.* Blood was obtained by venipuncture of the external jugular vein while rats were under light ether anesthesia. Blood was clotted at 4°C overnight and serum stored at −70°C until assay.

*Antibody assay.* IgG anti-collagen antibody was measured by an enzyme-linked immunosorbent assay (ELISA) system described by Rennard *et al.* (22) following minor modifications. Briefly, collagens or OVA were adsorbed to polystyrene microtiter plates (Nunc, Neptune, NJ) overnight at 4°C after being dissolved in 0.15 M potassium phosphate buffer, pH 7.6 (5 $\mu$g/ml). Plates were washed with 0.15 M NaCl (saline) containing 0.05% Tween 20. Samples of 100 $\mu$l of rat sera were added to duplicate wells after dilution in 0.1 M Tris-HCl buffered saline, pH 7.50, supplemented with 1% newborn calf serum and 0.5% Triton X-100. After a 1-hr incubation at 25°C, sera were removed, and the plates were washed. Peroxidase-conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, PA) was added at a predetermined dilution and incubated 90 min. After a final wash, 100 $\mu$l of orthophenylenediamine (OPD) substrate (40 mg OPD in 100 ml phosphate/citrate buffer, pH 5.0 and 40 $\mu$l of 30% $H_2O_2$) were added to each well. Colorimetric reactions of duplicate samples were read 1 hr later at 490 nm by using a Dynatech MR580 Microelisa Autoreader (Dynatech, Alexandria, VA) and were expressed as absorbance.

IgM anti-collagen antibody was measured by a modification of the procedure used to measure IgG. After rat sera were washed from the plates, rabbit anti-rat IgM (Miles Laboratories, Kankakee, IL) was added and incubated for 1 hr. The plates were then washed, and peroxidase-conjugated Fc-specific goat anti-rabbit IgG (Cappel Laboratories) was added as before. Ninety minutes later, the plates were washed and processed as described for IgG.

Serum dilutions of 1/100 and 1/1000, respectively, were used routinely to assay for IgM and IgG antibody in collagen-injected rats. Normal sera assayed at dilutions of 1/10–1/1000 yielded absorbance values of 0.003 to 0.012 for IgG and 0.040 to 0.050 for IgM. Sera from adjuvant-injected rats were assayed for anti-RII IgG at a dilution of 1/10.

This ELISA system is highly sensitive and able to detect affinity-purified rat anti-BII IgG at a concentration of < 10 ng/ml (absorbance 0.118); normal rat IgG (Cappel Laboratories) gave an absorbance reading of 0.013 when assayed at 10 ng/ml.

*Measurement of delayed-type hypersensitivity (DTH).* DTH was measured as the change in ear thickness (23) in millimeters (Δ mm) 48 hr after i.d. injection of 20 $\mu$g of BII dissolved in 0.02 ml of PBS. The opposite ear was injected with an equal volume of PBS and served as a control. Measurements were made with an engineer's micrometer and were expressed as the difference in thickness between collagen- and PBS-injected ears. Injection of BII into the ears of naive rats produced only negligible swelling (Δ 0.05 mm, i.e., 0.56 mm thickness preinjection; 0.61 mm 48 hr post-injection).

*Measurement of BII in venous blood after i.v. injection.* Clearance of immunoreactive BII from the circulation after i.v. injection was measured indirectly by ELISA by utilizing an inhibition assay. Rats were injected i.v. with 1 mg of BII, and blood was collected at regular intervals from the external jugular vein in heparinized syringes. A sample of 0.3 ml of whole blood was then mixed with 0.3 ml of diluted rat anti-BII serum. Equal amounts of blood from a noninjected donor or PBS served as controls. The mixtures of blood and antiserum were shaken gently on a platform rocker at 4°C for 1 hr. After centrifugation, 0.1-ml samples of the supernatants were added to microtiter plates coated with BII and were assayed as described. Sufficient amounts of blood were available from five bleedings to study the inhibitory activity of platelet-free plasma; normal plasma was used as a control in this instance. Inhibitory activity was not detected in whole blood or plasma obtained from normal rats. The percent inhibition was calculated by the formula:

% Inhibition

$$= \frac{\text{Absorbance anti-BII serum + BII-injected rat blood or plasma}}{\text{Absorbance anti-BII serum + normal rat blood or plasma}} \times 100$$

*Statistical analysis.* Statistical significance of data was determined by using the $\chi^2$ test with Yates correction or Student's *t*-test for nonpaired samples. Results are expressed as mean values ± standard error of the mean (SE).

## RESULTS

*CIA is specifically suppressed by i.v. injection of type II collagen.* To determine whether the clinical course of CIA or the immune response to type II collagen could be modified, rats were injected i.v. with a single 1-mg dose of native BII or CII collagen dissolved in 1 ml of PBS (groups I and II). Control rats (groups III through IV, respectively) received 1 ml of PBS or 1 mg of OVA or BI dissolved in 1 ml of PBS. After a 7-day rest, all rats were challenged with BII/IFA. The results in Table I show that the incidence of arthritis was significantly reduced only in rats pretreated with BII or CII. In group I, only two of 11 rats developed CIA after challenge. One rat had mild, transient arthritis affecting a single hind limb (MAI, 3), and the other rat had severe arthritis involving 3 limbs (MAI, 11). None of the rats in group II given i.v. CII developed arthritis after challenge with BII/IFA. Six rats from group I that failed to develop CIA had their ankles examined histologically for evidence of subclinical arthritis and were found to be normal.

Rats in groups IV and V pretreated with antigens other than type II collagen were not protected, and arthritis developed in both groups at a similar incidence, severity, and day of onset as PBS controls (group III). These values for groups III–V are comparable to those previously reported in rats challenged with BII/IFA without i.v. pretreatment (3, 4, 9).

Anti-collagen antibody levels corresponded directly with the presence or absence of arthritis. IgM and IgG antibodies to BII and IgG cross-reactive with homologous RII were significantly depressed in rats pretreated with BII or CII (groups I and II). Suppression of anti-BII antibody was also examined by titering sera obtained 28 days after challenge from eight nonarthritic and eight arthritic rats (groups I and III, respectively). The rats in group I had a mean titer of 200 (range < 50 to 800) *vs* 51,200 (range 12,800 to 104,800) for arthritic rats in group III ($P < 0.001$) when titer was defined as the first serum dilution yielding an absorbance value of < 0.075. Finally, the mean anti-BII IgG value of nonarthritic rats from group I was compared with that of the five nonarthritic rats from groups III, IV, and V and was found to be significantly lower (0.047 ± 0.049 *vs* 0.581 ± 0.089, respectively, day 28, $P < 0.001$). The two rats in group I that developed arthritis after pretreatment had antibody levels that correlated with the severity of their arthritis (0.260 and 0.647). DTH responses measured by skin test reactivity to BII were also significantly depressed by i.v. pretreatment with BII (Table I).

*Intravenous pretreatment with BII does not inhibit immunity to unrelated antigens.* To determine if suppression of immunity to BII was specific and not due to an inhibitory or toxic property peculiar to type II collagen, six groups of rats were pretreated with BII or PBS before being sensitized with BI, OVA, or BII (Table II). The protocol for this study was identical to the one previously described except that rats in group X were challenged first with BII in CFA.

Pretreatment with BII suppressed immunity to only BII and

TABLE I

*Suppression of CIA and immunity to type II collagen by i.v. injection of BII or CII*[a]

| | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| I.v. Pretreatment | BII | CII | PBS | OVA | BI |
| Incidence of CIA | 2/11[b] | 0/10[c] | 8/10 | 9/10 | 8/10 |
| X Day of onset | 16.0 ± 2.0 | 0 | 13.4 ± 0.8 | 12.4 ± 0.3 | 12.3 ± 0.5 |
| MAI | | | | | |
| Arthritic rats | 7.0 ± 4.0 | 0 | 6.9 ± 0.9 | 8.4 ± 1.2 | 8.1 ± 0.5 |
| Per group | 1.3 | 0 | 5.5 | 7.6 | 6.5 |
| IgM to BII day 16[d] | 0.088 ± 0.022[c] | ND[e] | 0.363 ± 0.026 | 0.487 ± 0.055 | 0.310 ± 0.042 |
| IgG to BII day 16 | 0.028 ± 0.012[c] | ND | 0.241 ± 0.026 | 0.374 ± 0.050 | 0.307 ± 0.070 |
| IgG to BII day 28 | 0.190 ± 0.057[c] | 0.162 ± 0.048[c] | 0.710 ± 0.051 | 0.750 ± 0.07 | 0.660 ± 0.087 |
| IgG to RII day 28 | 0.114 ± 0.059[c] | 0.130 ± 0.045[c] | 0.710 ± 0.050 | 0.748 ± 0.076 | 0.664 ± 0.087 |
| DTH to BII day 30[f] | 0.32 ± 0.060[c] | ND | 1.15 ± 0.13 | ND | ND |

[a] Seven days before i.d. challenge with BII/IFA, rats were pretreated i.v. with 1 mg of BII, CII, OVA, or BI dissolved in 1 ml of PBS. Rats in group III received 1 ml of PBS i.v. Challenge is described in *Materials and Methods*.

[b] $P < 0.01$ vs groups III–V.

[c] $P < 0.001$ vs groups III–V.

[d] Antibody was measured by ELISA and expressed as absorbance. Values shown are the mean absorbances of arthritic and nonarthritic rats in each group ± SE. Antibody to BII was not detected in normal sera or the sera of six rats given i.v. BII 14 days earlier but not challenged with BII/IFA.

[e] Not done.

[f] DTH was measured by injecting on ear i.d. with 20 μg BII and the other with PBS. Values shown represent Δ in swelling in millimeters of study ear minus millimeters of control ear 48 hr with i.d. injection. DTH studies were performed on a separate group of rats (groups XV and XVIII, Table IV) pretreated i.v. with 1 mg of BII or 1 ml of PBS before challenge with BII/IFA. Only one of 10 BII-pretreated rats developed CIA vs eight of 10 controls.

TABLE II

*Studies showing the specificity of suppression induced by BII*[a]

| Group | I.v. Pretreatment Day −7 | Challenge Day 0 | Challenge Day +7 | Incidence of Arthritis | IgG Response to Respective Immunogen[b] |
|---|---|---|---|---|---|
| VI | BII | BI/IFA | BI/IFA | 0/8 | 1.034 ± 0.35[c] |
| VII | PBS | BI/IFA | BI/IFA | 0/8 | 0.972 ± 0.040 |
| VIII | BII | OVA/IFA | OVA/IFA | 0/6 | 0.425 ± 0.047[c] |
| IX | PBS | OVA/IFA | OVA/IFA | 0/6 | 0.340 ± 0.036 |
| X | BII | BII/CFA | BII/IFA | 0/10 | 0.036 ± 0.008[d] |
| XI | PBS | BII/IFA | BII/IFA | 8/10 | 0.753 ± 0.036[e] |

[a] Rats were pretreated i.v. with 1 mg of BII or 1 ml of PBS 7 days before immunization with BI, OVA, or BII in IFA.

[b] Blood was collected 28 days after first challenge. Values are mean absorbance per group ± SE.

[c] Not significant vs PBS control.

[d] $P < 0.001$, group X vs XI.

[e] IgG cross-reactive to RII was 0.037 ± 0.008 and 0.750 ± 0.038 for groups X and XI, respectively.

TABLE III

*Effect of time interval between i.v. injection of BII and challenge on the course of CIA*[a]

| Group | Injection (i.v.) | Day of i.v. Injection (Challenge Day 0) | Incidence of Arthritis | IgG Response[b] Day +28 |
|---|---|---|---|---|
| XI | BII | −32 | 1/5 | ND |
| | PBS | | 4/6 | ND |
| XII | BII | −7 | 2/11[c] | 0.190 ± 0.057[d] |
| | PBS | | 8/10 | 0.710 ± 0.051 |
| XIII | BII | −4 | 1/10[c] | 0.040 ± 0.012[d] |
| | PBS | | 8/10 | 0.717 ± 0.064 |
| XIV | BII | +7 | 1/10[c] | 0.264 ± 0.061[d] |
| | BI | | 7/10 | 0.850 ± 0.120 |

[a] Rats were injected i.v. with 1 mg of BII before or after challenge as shown. Control rats received an equal 1-ml volume of PBS at the same time, except for group XIV, which was injected i.v. at day +7 with 1 mg of BI solubilized in PBS. Challenge was performed as described earlier. ND, not done.

[b] Mean absorbance ± SE.

[c] $P < 0.01$ vs PBS control.

[d] $P < 0.001$ vs PBS- or BI-injected controls.

had no effect on IgG responses to either BI or OVA. Moreover, the suppression induced by i.v. pretreatment with BII could not be overcome by using CFA, a more potent immunoadjuvant.

BI was found to be a potent immunogen producing absorbance values greater than BII. This finding differs from an earlier report in which heterologous chick type I collagen was described as a poor immunogen in the rat when emulsified with IFA (2). The discrepancy between that study and ours may reflect either differences in the sensitivity of hemagglutination and ELISA assays for antibody to type I collagen or, alternatively, differences in the immunogenicity of these type I collagens.

*Importance of time interval between i.v. injection of BII and i.d. challenge.* The incidence of different injection schedules on the course of CIA and immunity to BII is shown in Table III. The incidence of CIA was reduced significantly when 1 mg of BII was given i.v. 32, 7, or 4 days before challenge or 7 days after. Anti-BII IgG was suppressed in all groups given BII as compared with controls given PBS ($P < 0.001$). Arthritis and antibody to BII were observed in a few rats (5 of 36) given i.v. BII before or after challenge, however, the day of onset, severity, and IgG values were variable. In subsequent studies suppression of CIA and antibody to BII has been virtually 100% effective suggesting that occasional failures may have resulted from technical problems. (Data not shown).

The course of rats given i.v. BII 7 days after challenge (group XIV) was different from those pretreated with BII. Nine of 10 rats in group XIV became acutely ill within 24 hr of i.v. injection, developed proteinuria, and lost weight. Clinical changes and proteinuria were not seen in 10 control rats given BI at the same dose and time. The kidneys of one rat examined histologically 7 days after the onset of proteinuria disclosed a mild glomerulonephritis suggesting injury by BII-immunoglobulin complexes. Immunofluorescence studies, however, were not performed. Clinical changes and proteinuria were not found in a group of six rats given i.v. BII alone or 10 rats given i.v. BII and then challenged 7 days later. (Data not shown). Histologic examination of the kidneys of six untreated arthritic rats disclosed no abnormality.

Finding that arthritis could be suppressed after challenge, we attempted to inhibit active arthritis. A group of six rats with severe arthritis, immunized 32 days earlier with BII/IFA, was chosen for study. After an initial bleeding, rats were rested for 2 days and then given 1 mg of BII i.v.; blood was collected 2 and 21 days later. No detectable change in the severity of arthritis was noted after collagen injection. Anti-BII IgG values for the three bleedings were 0.810 ± 0.089, 0.512 ± 0.106, and 0.813 ± 0.137, respectively, indicating only a slight, possibly transient fall in IgG.

Proteinuria was not detected in rats given BII i.v. 32 days after immunization. This finding suggests that the ratio of antigen and antibody may determine the pathogenicity of BII-immunoglobulin complexes formed *in vivo*.

*Importance of dose and route of injection of BII.* Suppression

of CIA was dependent on the amount of BII given and the route of injection as shown in Table IV. Pretreatment was performed as before except i.v. injections were given 4 days before challenge. This was done for convenience because 4- and 7-day pretreatment schedules were quite effective in suppressing arthritis and immunity to BII.

Only one of 10 rats given 1 mg of BII i.v. developed CIA in contrast to eight of 10 controls pretreated with 1 ml of PBS ($P < 0.01$). Rats pretreated with 0.1 or 0.01 mg of BII in equal volumes of PBS developed arthritis at an incidence and severity comparable to controls though at later dates of onset. Rats pretreated with 1 mg of BII s.c. also developed arthritis similar to controls.

Sera of nonarthritic rats, pretreated i.v. with 1 mg of BII, contained only traces of IgG specific to BII as compared with PBS-injected controls. The one arthritic rat in this group developed an IgG value of 0.677, which was comparable with arthritic controls. Sera of rats pretreated with 0.1 or 0.01 mg of BII contained intermediate amounts of IgG suggesting a slight inhibition of response consistent with the delayed onset of arthritis.

*Adjuvant arthritis is not suppressed by pretreatment with BII.* To determine if AIA could be suppressed by i.v. pretreatment with BII and if rats with AIA produce antibody to RII, an additional study was done. Adjuvant arthritis was induced in three groups of rats by a single i.d. injection of mycobacteria suspended in oil. Groups one and two each contained 10 rats and received i.v. pretreatment with 1 mg of BII or 1 ml of PBS 4 days before i.d. injection with adjuvant. Heterologous BII was used instead of homologous RII because of BII's strong cross-reactivity with RII and ability to suppress immunity to RII in rats immunized with BII/IFA and BII/CFA (Tables I and II). Moreover, in preliminary studies, BII was found to be more effective than RII in inducing and suppressing arthritis (unpublished observations). Group three contained 26 rats and received no i.v. pretreatment before injection with adjuvant. The latter group was bled weekly from day 14 through day 35 to provide sera for antibody studies. Severe arthritis developed in all 46 rats.

Pretreatment with BII affected neither the incidence, severity, time of onset of AIA, nor the incidence of ear nodules or spondylitis, compared with rats pretreated with PBS (Table V). Furthermore, anti-RII IgG was not detected at a 1/10 dilution in any of the sera assayed by a sensitive ELISA system that readily detected antibody in rats with CIA.

*Clearance of immunoreactive BII from peripheral blood.* The rate solubilized BII was cleared from the circulation after i.v. injection was indirectly determined by ELISA utilizing an antibody inhibition assay. Two normal rats were given an i.v. injection of 1 mg of BII, and venous blood was collected in heparinized syringes at regular intervals.

The upper curve in Figure 1 shows the clearance of BII from whole blood. Two distinct slopes are apparent, an early one lasting between 15 and 60 min, which shows rapid clearance; and a later one lasting between 2 and 8 hr, which shows a more gradual clearance of BII. The lower curve shows the clearance of BII from platelet-free plasma, which was complete within 2 hr. These data imply that BII present 2 hr after i.v. injection was bound to cellular elements. In earlier studies, performed *in vitro*, we found that BII spontaneously bound to splenocytes and erythrocytes (unpublished observation) suggesting that a similar process may occur *in vivo* after i.v. injection.

## DISCUSSION

In this report, we have shown that soluble native type II collagen induces and suppresses CIA in rats depending on the mode of its administration. Rats immunized i.d. with BII in IFA developed arthritis at an incidence of 70 to 90% and developed a strong immune response to type II collagen. When immunization was preceded or followed shortly by an i.v. injection of soluble type II collagen, however, the incidence of arthritis was reduced to 0 to 18%, and immunity to collagen was significantly suppressed.

Our data suggest that CIA was prevented by the induction of immune tolerance. The suppression of immunity was antigen specific, dependent on the i.v. route of injection, and related to the dose of BII. The antigen-specific nature of suppression was shown in two studies. First, neither arthritis nor immunity to BII were suppressed by PBS, BI, or OVA given i.v. before challenge with BII/IFA. Conversely, it was also found that i.v. pretreatment with BII had no effect on immunity to BI or OVA when rats were immunized with BI/IFA or OVA/IFA. Both studies demonstrate a) that the suppression of CIA, like its induction, is critically dependent on collagen type, and b) that nonspecific mechanisms of suppression, i.e., stress (17), and the activation of antigen-independent suppressor T-cells (24) or macrophages (25), were not triggered by i.v. injected collagen. Lastly, we found that suppression of CIA was not dependent on the species source of type II collagen used for pretreatment. Intravenous injection of BII or CII was effective in suppressing arthritis and anti-RII IgG in rats challenged with BII/IFA. This data suggests that bovine- and avian-derived type II collagens share significant antigenic homology with homologous RII.

On comparing our results with previous models of tolerance, the rapid clearing of BII from the blood after i.v. injection is in sharp contrast with reports that other soluble proteins used as tolerogens circulate for weeks after i.v. injection (26). The rapid clearance of BII demonstrates that collagen need not circulate for long periods to induce or maintain suppression.

Our studies on inducing tolerance after immunization are consistent with the work of other investigators who examined different antigens. We found, as did Sanfillippo and Scott (26), that immunity could be suppressed in an antigen-specific manner when antigen was administered i.v. 7 days after immunization. No suppression of arthritis or immunity to BII, however, was seen when i.v. injection was delayed 32 days after immunization.

TABLE IV

*Dose response of BII, route of injection, and the course of CIA*[a]

| Group | Dose of BII per Rat | Route | Incidence of Arthritis | MAI | | Mean Day of Onset ± SE | IgG Response at Day 28 ± SE |
|---|---|---|---|---|---|---|---|
| | | | | Arthritic rats | Per group | | |
| XV | 1.0 mg | i.v. | 1/10[b] | 3 | 0.3 | 35 | 0.040 ± 0.012[c] |
| XVI | 0.1 mg | i.v. | 7/10 | 6.0 ± 0.7 | 4.2 | 25 ± 2.8[c] | 0.412 ± 0.070 |
| XVII | 0.01 mg | i.v. | 9/10 | 5.4 ± 1.4 | 4.7 | 20.2 ± 1.3[c] | 0.499 ± 0.107 |
| XVIII | PBS | i.v. | 8/10 | 6.8 ± 0.8 | 5.4 | 13.1 ± 1.0 | 0.717 ± 0.064 |
| XIX | 1.0 mg | s.c. | 6/10 | 6.2 ± 0.9 | 3.7 | 18.7 ± 4.5 | ND |

[a] Rats were injected i.v. with 1.0, 0.1, or 0.01 mg of BII solubilized in 1 ml of PBS. Controls in group XVIII received 1 ml PBS. Rats were challenged 4 days later as described. ND, not done.

[b] $P < 0.01$ vs group XVIII.

[c] $P < 0.001$ vs group XVIII.

TABLE V
*Pretreatment with BII does not suppress adjuvant arthritis*

| | Intravenous Pretreatment[a] | | |
|---|---|---|---|
| | BII | PBS | None |
| Incidence of AIA | 10/10 | 10/10 | 26/26 |
| MAI | 14.7 ± 0.6 | 15.0 ± 0.4 | 12.4 ± 0.6 |
| Arthritic limbs/group | 39/40 | 40/40 | 95/104 |
| Mean day of onset | 11.9 ± 0.5 | 11.1 ± 0.3 | 11.9 ± 0.2 |
| Incidence of: | | | |
| Ear nodules | 10/10 | 10/10 | 26/26 |
| Spondylitis[b] | 9/10 | 10/10 | NR[c] |

[a] Rats in each group received i.v. pretreatment with 1 mg of BII or 1 ml of PBS 4 days before i.d. injection with 250 $\mu$g of ground mycobacteria suspended in oil.
[b] Spondylitis was defined as kyphotic deformity of the spine or ankylosis of the tail distal to the site of adjuvant injection.
[c] NR, not recorded.

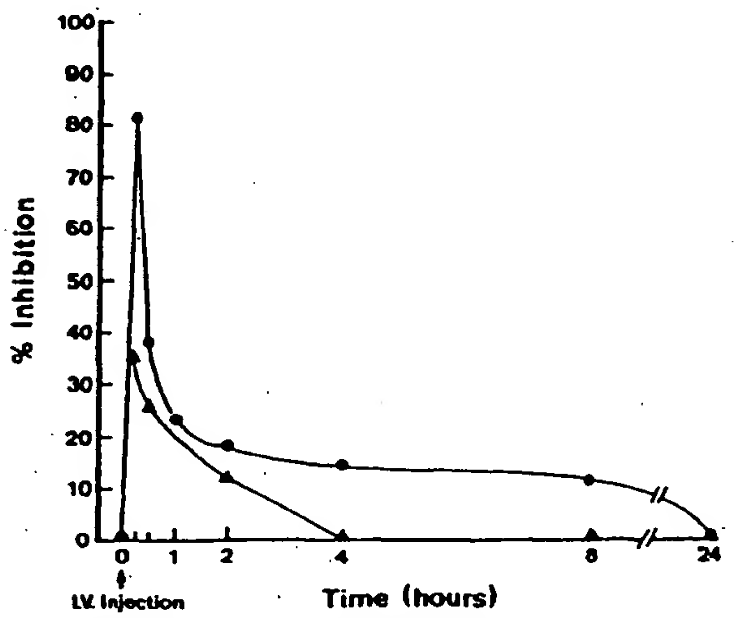


*Figure 1.* Clearance of immunoreactive BII from peripheral venous blood of a normal rat injected i.v. with 1 mg solubilized BII. Assay for BII was performed by measuring the inhibitory activity of circulating BII against anti-BII IgG in an ELISA system. ●, represent the inhibitory activity of whole blood; ▲, the inhibitory activity of platelet-free plasma.

Other workers have also reported difficulty in inducing tolerance 2 to 4 wk after immunization (27, 28).

Suppression of CIA with type II collagen has been reported by two other groups. Schoen *et al.* (14) found that the incidence of CIA in rats could be reduced, in an antigen-specific manner, by i.v. injection of CII-coupled spleen cells. A series of four injections reduced anti-CII IgG levels by ≈50% as measured by radioimmunoassay. This treatment, however, had no effect on hemagglutination titers or DTH reactivity to CII. In comparison, immunosuppression induced by i.v. injection of soluble collagen, which suppressed antibody and DTH, was greater than that reported by Schoen and co-workers. Differences between their study and ours may be attributable to the form or amounts of collagen injected.

Staines *et al.* (18) recently found that CIA and anti-RII IgG were suppressed by treating rats with i.v. injections of pig type II collagen (100 $\mu$g/rat) and immune serum (1 ml/rat) given 8 and 9 days before i.d. challenge, respectively. Pretreatment with only collagen or serum produced a modest suppressive effect, whereas the combination was quite effective. Our study also showed a modest degree of antibody suppression when 100 $\mu$g of type II collagen were used for pretreatment. Although these investigators did not study the effects of larger doses of collagen, our work shows that at least 1 mg of type II collagen is needed to produce significant suppression.

The importance of immunity to type II collagen in the pathogenesis of CIA has been amply demonstrated (1–21). Work from this laboratory (2, 9) and others (6, 7) has shown that immunizing rats with homologous or heterologous type II collagens, prepared from a variety of species, induces arthritis and immunity to homologous RII. The critical importance of antibody to type II collagen in the development of CIA is now clearly established. Morgan *et al.* (12) first suggested this by showing that arthritis did not appear in BII-immunized rats as long as serum C was depleted. This finding and the work of Schoen *et al.* (14) indirectly emphasized the importance of humoral immunity in CIA and questioned the role of cell-mediated immunity (CMI) to collagen in CIA. Recently, studies from this laboratory provided direct evidence for an antibody-mediated pathogenesis of CIA by showing that IgG specific for type II collagen produces arthritis when passively transferred to normal recipients (15, 16).

Several investigators have suggested that autoimmunity to collagen might play a role in the pathogenesis of AIA (29–33). Steffen and co-workers were the first to report that adjuvant arthritic rats developed weak DTH to denatured (30) and native (31) type I collagen. Trentham *et al.* (32) reported CMI to native and denatured homologous type I and II collagens in rats with AIA. Recently, Holoschitz *et al.* (33) isolated an effector T cell line from adjuvant arthritic rats that proliferated strongly to mycobacteria and weakly to RII. They found this cell line effective in transferring or vaccinating against AIA. A T cell line reactive only to RII from rats immunized with RII/IFA was ineffective in transferring CIA. Furthermore, Schoen *et al.* (34) showed that s.c. injection of RII-coupled spleen cells induced RII-specific CMI in rats but neither arthritis nor antibody to RII. These findings and those previously reported by others (12, 14) suggest that CMI to type II collagen alone is not sufficient to induce arthritis. These data, however, do not exclude the importance of Th cells in the genesis of immunity to type II collagen, a T-dependent antigen, nor the possibility that CMI may contribute to tissue injury once arthritis is initiated (35).

Antibodies to native and denatured type I and II collagens, measured by hemagglutination, have been reported in ≈40% of rats with AIA (32). The authors of that report, however, were unable to conclude whether anti-collagen antibodies represented a primary or secondary event. In contrast to CIA, in which antibodies levels are high, appear before the onset of arthritis, and react primarily to type II collagen, antibody titers described in AIA were frequently low, appeared after the onset of arthritis, and reacted well to type I and II collagens. These data suggest to us that immunity to collagen in adjuvant disease represents a secondary response produced by injury of articular tissues or granuloma formation. In our studies, we were not able to detect antibody to type II collagen in severe AIA by using a sensitive ELISA system.

Other reports provide data suggesting that CIA and AIA have dissimilar primary pathogenetic mechanisms. We first suggested this possibility when we found that type II collagen lacked adjuvant activity (36), a property intimately associated with the ability of natural (37) and synthetic substances to induce adjuvant arthritis (38, 39). Our work has been since confirmed by Iizuka and Chang (40). Furthermore, these investigators showed that AIA could be suppressed by pretreatment with a subarthritogenic dose of mycobacteria given s.c. before immunization with adjuvant, whereas the same procedure had no effect on CIA. Data presented here show, conversely, that i.v. pretreatment with BII suppressed CIA and immunity to BII and RII but had no suppressive effect on AIA. This observation and the absence of anti-RII IgG in adjuvant arthritis further support the conclusion that immunity to type II collagen is not central to the pathogenesis of AIA.

In conclusion, studies reported here show that CIA in rats and

immunity to type II collagen can be suppressed in an antigen-specific manner by i.v. injection of native type II collagen. This data further supports the hypothesis that CIA is mediated by immunity to type II collagen. Furthermore, attempts to suppress AIA by i.v. injection of collagen and to demonstrate anti-collagen antibody in AIA were not successful. These findings suggest that CIA and AIA, despite some phenotypic similarities, are distinct entities mediated by different primary pathogenic mechanisms.

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# Control of Cellular and Humoral Immune Responses by Peptides Containing T-cell Epitopes

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T cells are known to recognize antigen in the form of peptides bound to major histocompatibility complex (MHC)-encoded class II molecules (Babbit et al. 1985; Schwartz et al. 1985; Buus et al. 1987; Guillet et al. 1987). Experiments with MHC-encoded class II molecules in planar membranes show that the binding of the T-cell receptor (TCR) to the peptide/MHC complex may not be sufficient for activation of the responding T cells (Quill and Schwartz 1987). One signal to the T cells is the binding of the TCR/CD3 complex to the antigen/MHC. As proposed by Bretscher and Cohn (1970), lymphocytes may be tolerized unless they see a second signal. This second signal for T cells can be provided in principle by the antigen-presenting cell (APC), either through the secretion of soluble cytokines or by the interaction of molecules on the APC and T-cell surface. To test this hypothesis, we exposed mice to peptides containing known T-cell epitopes without adjuvant in order to expose the T cells only to the first (TCR) signal.

The biological relevance of the existence of various APC types such as macrophages, B cells, and dendritic cells in vivo is still unclear. As mentioned above, the second signal necessary for the activation of T cells may be provided by APCs (Quill and Schwartz 1987). It is also possible that different types of APCs may provide different second signals and may thus affect the quantitative and qualitative outcome of immune responses. In this paper, the responding T cells from mice immunized with the amino-terminal fragment of λ repressor *c*I 1-102 using different immunization protocols are characterized with respect to the distribution of T-cell epitopes recognized, as well as their functional phenotypes upon presentation of T-cell epitopes by different APCs in vitro.

The protein context of a peptidic T-cell epitope also influences the T-cell response specific for that epitope (Shastri et al. 1986). Thus, the immune response to a T-cell epitope may be hidden when it is covalently linked to another T-cell epitope. This can be due, for example, to competition between epitopes for binding to the MHC peptide-binding site (Guillet et al. 1987). Antigen processing can also explain differential expression of epitopes, depending on the context. Thus, neighboring or distal residues could influence processing so that a particular T-cell epitope is unavailable to bind MHC. We compare the activity of an ovalbumin-derived peptide epitope that is suppressed when covalently linked to an epitope of greater immunogenicity in an antigenic and tolerogenic system.

## MATERIALS AND METHODS

*Animals.* Strains BALB/cByJ, C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

*Cell cultures and assay conditions.* All cultures and assays were performed according to established procedures (Lai et al. 1987).

*Antigens.* Bacteriophage λ repressor *c*I protein fragment p1-102 and synthetic peptides were prepared as described previously (Lai et al. 1987). p12-26$^{N14}$ is residues 12–26 of *c*I with asparagine substituted for aspartic acid at position 14.

*Cell lines.* BW5147.G.4.Oau$^{R}$.1($\alpha$-,$\beta$-) was a gift from W. Borne. A20.2J (I-A$^{d}$, I-E$^{d}$) was a gift from J. Kappler and P. Marrack. CTLL.2 was a gift from D. Raulet. CT.4S (Hu-Li et al. 1989) and 11B11 (anti-IL-4) (Ohara and Paul 1985) were a gift from W. Paul. S4.B6 (anti-IL-2) was a gift from T. Mosmann. Class II MHC L-cell transfectants RT 2.3.3H (I-A$^{d}$) and RT 10.3H2 (I-E$^{d}$) were a gift from R. Germain.

*Antibodies.* Monoclonal antibodies were produced by harvesting cell supernatants from B-cell hybridomas. Supernatants were centrifuged and sterile filtered to remove any residual cells. 11B11 supernatant was used at 10%, and S4B6 supernatant was used at 50%.

*Antisera.* BALB/c mice were tolerized by injection of p12-26 i.v. in saline, or saline alone as a control. They were immunized 10 days after the first tolerization with p12-26 i.p. (50 μg emulsified in complete Freund's adjuvant [CFA]). Mice received a booster immunization 14 days after the primary injection of p12-26 i.p. (50 μg emulsified in incomplete Freund's adjuvant [IFA]). Mice were bled 7 days after the first immunization and 8 days after the booster immunization (data shown). Antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (Good et al. 1988).

*Isolation of T-cell hybridomas.* Mice were immunized either s.c. at the base of the tail and in both thighs or i.p. with 100 $\mu$g of bacteriophage $\lambda$ repressor p1-102 in either CFA, IFA, or alum. After 7 days, draining lymph nodes were removed from mice with s.c. immunizations, and spleen cells were removed from those with i.p. immunizations. Cells were stimulated in vitro with p1-102 at 50 $\mu$g/ml for 2 days before fusion with BW5147. Fusion hybrids were prepared according to established procedures (Gefter et al. 1977).

All the T-cell hybridomas reported here have been subcloned at least once by limiting dilution method (Walker et al. 1982). 9C127 and 1E1 are hybridomas made from BALB/c mice. 3D054.8 is a clone specific for OVA(325-336).

*Tolerization of adult mice.* Mice 4–8 weeks old were injected i.v. in the tail vein with 300 $\mu$g of deaggregated peptide dissolved in 100 $\mu$l of saline on day 0. After 5 days, another 300 $\mu$g of peptide was injected i.v. On day 10, the mice were immunized s.c. with peptide in CFA as described above.

*Lymphokine assays.* IL-2 assays were performed as described previously (Lai et al. 1987). Assay for IL-4 was performed using the IL-4-dependent cell line CT.4S according to the method of Hu-Li et al. (1989), with the slight modification that tritium incorporation in DNA was measured 6 hours after the addition of 1 mCi of tritiated thymidine.

*Lymphocyte proliferation assays.* T-lymphocyte proliferation assays were performed according to methods described by Lai et al. (1987).

## RESULTS

We have previously shown that the T-cell response in BALB/c mice to the amino-terminal fragment of $\lambda$ repressor (p1-102) is predominantly directed to a synthetic peptide (p12-26) containing residues 12–26 (Guillet et al. 1987; Roy et al. 1989). Proliferation of p1-102-immunized T cells is equivalent in response to either p1-102 or p12-26, and over 90% of BALB/c-derived T-cell hybrids made against p1-102 respond to p12-26. In addition, p12-26 can serve to recruit help, since immunization of mice with p12-26 produces anti-p12-26 antibodies (Roy et al. 1989). When BALB/c mice are exposed to p12-26 by intravenous injection, they become unresponsive to later immunization with p12-26 s.c. in CFA. The same is true for p12-26$^{N14}$, a related peptide that cross-reacts with p12-26 (Fig. 1). The tolerization procedure decreases lymphocyte proliferation (Fig. 1a) and almost eliminates IL-2 secretion (Fig. 1b) of draining lymph node cells. The effect is epitope-specific, since i.v. injection of saline alone or of unrelated peptides has no effect. In addition, C57BL/6 mice can be tolerized to their immunodominant epitope in p1-102, p73-88 (data not shown). This tolerization protocol also eliminates T-cell help, since treated mice can no longer produce antibodies to p12-26 (Table 1).

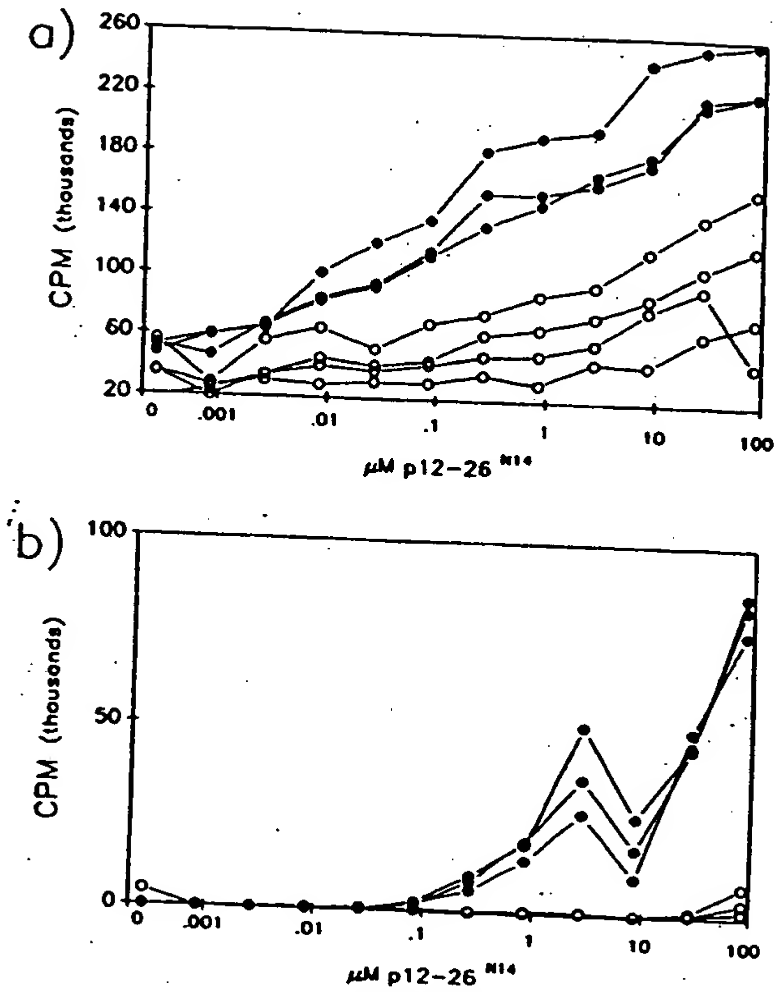


**Figure 1.** Response of lymphocytes from BALB/c mice immunized with p12-26$^{N14}$ s.c. (○) Mice tolerized by two i.v. injections of p12-26$^{N14}$; (●) control mice. (*a*) Proliferation; (*b*) IL-2 secretion as measured by proliferation of CTLL cells.

The reduced response cannot be due to a sudden high concentration of soluble antigen inactivating T cells, since the same tolerization effect can be seen when antigen is injected i.p. in IFA 10 days before immunization (data not shown).

The tolerance effect lasts for at least 6 weeks (Table 2). The T-cell proliferation is initially reduced to only 20% of that of immunized mice, whereas the IL-2 secretion is reduced even more, to less than 5%. After 42 days, the IL-2 secretion of tolerized T cells seems to have increased to 15% of that of control T cells, whereas only one of two mice tested have reduced proliferation. Studies are continuing to investigate further the duration of the T-cell nonresponsiveness.

The T-cell tolerance could be due to the inactivation of antigen-specific T cells or the induction of suppressor

**Table 1.** Antibody Response to p12–26

| Mouse | Pre-bleed ($\mu$g/ml) | Immunized ($\mu$g/ml) |
|---|---|---|
| Tolerized | <2 | 19 |
| Tolerized | 3 | 12 |
| Tolerized | 3 | 15 |
| Tolerized | 3 | 5 |
| Control | 2 | 528 |
| Control | 4 | 378 |
| Control | 2 | 99 |
| Control | 3 | 363 |

Table 2. Persistence of Tolerization Effects

| Day immunized | % of Control | |
|---|---|---|
| | IL-2 | proliferation |
| 1 | 3, 2 | 19, 19 |
| 5 | 1, 2 | 28, 38 |
| 9 | 6, 20 | 14, 39 |
| 41 | 11, 17 | 17, 103 |

BALB/c mice were i.v. tolerized on day −5 and day 0 p12-26. Mice were immunized at various times thereafter and tested for specific proliferation and IL-2 secretion 7 days later.

T cells. To test the latter possibility, mixtures of lymphocytes from tolerized and nontolerized mice were compared with mixtures of lymphocytes from nonimmunized and nontolerized mice. It is expected that if tolerization were due to the induction of suppressor T cells, then the suppressor T cells present in tolerized mice should suppress the response of immunized T cells, whereas mixing with nonimmunized T cells should have no effect. As seen in Figure 2, the proliferation of immunized cells mixed with tolerized cells was the same as that of immunized cells mixed with nonimmune cells. If anything, the response was a bit higher, as could be expected, because tolerized cells still proliferate at 20% of the control level. Furthermore, the proliferation clearly rises as more immunized cells are added, until, with 99% control immunized cells and only 1% tolerized or nonimmunized cells, the response is normal. The same result was obtained with IL-2 secretion (data not shown).

We then investigated the immune response to a molecule in which two T-cell epitopes were combined. The two epitopes chosen were residues 12–26 (p12-26) of λ repressor and residues 325–336 (OVA-D) of egg ovalbumin, with valine residue 327 changed to aspartic acid to reduce binding to I-$A^d$ (Sette et al. 1987). Both epitopes are I-$A^d$-restricted, and p12-26 and ova325-336 are immunodominant in their respective proteins when injected into BALB/c mice (Guillet et al. 1987; Shimonkevitz et al. 1987). We synthesized a long joint peptide, 12-26-GPG-OVA-D, containing p12-26 linked to OVA-D by a glycine-proline-glycine bridge (see Table 3). T cells from BALB/c mice immunized with OVA-D proliferate and secrete IL-2 when stimulated in vitro with OVA-D. Similarly, T cells from mice immunized with p12-26 proliferate and secrete IL-2 in response to either p12-26 or the joint peptide (Fig. 3a). In contrast, lymphocytes from mice immunized with the long peptide respond in vitro to the long peptide and to p12-26, but not to OVA-D (Fig. 3e). Thus, the OVA-D epitope is hidden when present within the joint peptide. The basis for this result could be easily explained by competition between the two epitopes for binding to MHC-encoded class II molecules and perhaps also to a third epitope created at the junction of the two fused epitopes. This epitope suppression is not due to the inability of BALB/c-presenting cells to present OVA-D from within the long peptide, since the long peptide stimulates the ova325-336-restricted T-cell hybridoma 3DO54.8 when presented on I-$A^d$-transfected L cells (Fig. 4a). I-$A^d$-transfected L cells (Fig. 4b) and fixed or live H-$2^d$ A20 cells (Fig. 4c) can also present the joint peptide to p12-26-specific hybridomas 9C127(I-$A^d$-restricted) and 1E1 (I-$E^d$-restricted). Thus, processing of the joint peptide is not required, at least for presentation of the p12-26 epitope in vitro.

Mice immunized with p12-26, which 10 days earlier had been tolerized by i.p. injection with either p12-26 (Fig. 3b) or the joint peptide (Fig. 3d) in IFA, have diminished responses, whereas mice given saline or OVA-D-tolerized mice proliferate normally in response to either p12-26 or the joint peptide (Fig. 3a,c). Similarly, mice immunized with the joint peptide that were previously tolerized with the joint peptide cannot respond to either p12-26 or the joint peptide (Fig. 3h), and those tolerized to p12-26 cannot respond to p12-26 (Fig. 3f) but can respond to a lesser extent to the joint peptide, possibly because T cells specific for the junction were not tolerized. Control mice tolerized to OVA-D (Fig. 3g) or not tolerized (Fig. 3e) respond to both p12-26 and the joint peptide. Mice immunized with OVA-D that were previously tolerized with OVA-D cannot respond to OVA-D or the joint peptide (Fig.

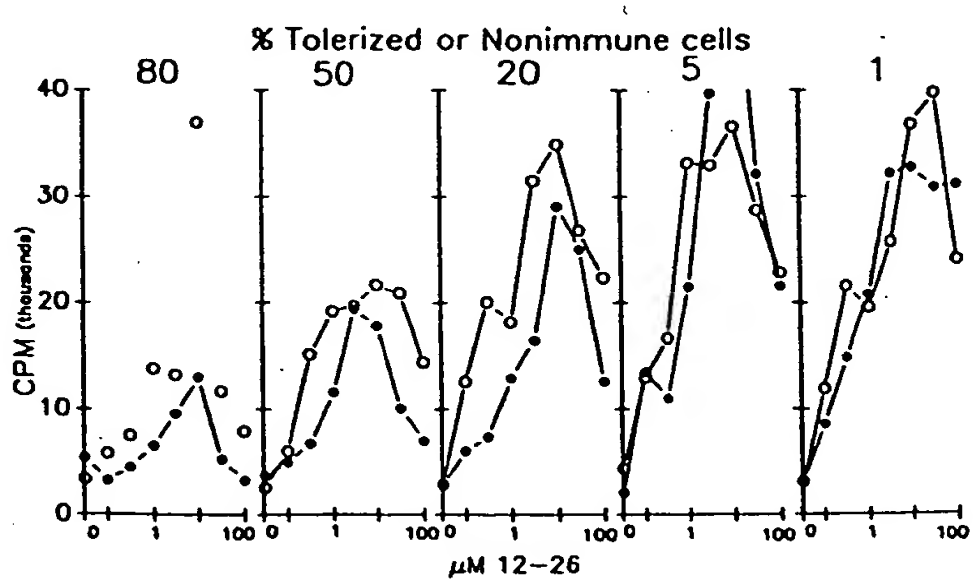


Figure 2. Proliferation of lymphocytes from mice immunized with p12-26. Lymphocytes from control mice were mixed with various percentages of lymphocytes from either tolerized mice (O) or nonimmunized mice (●).

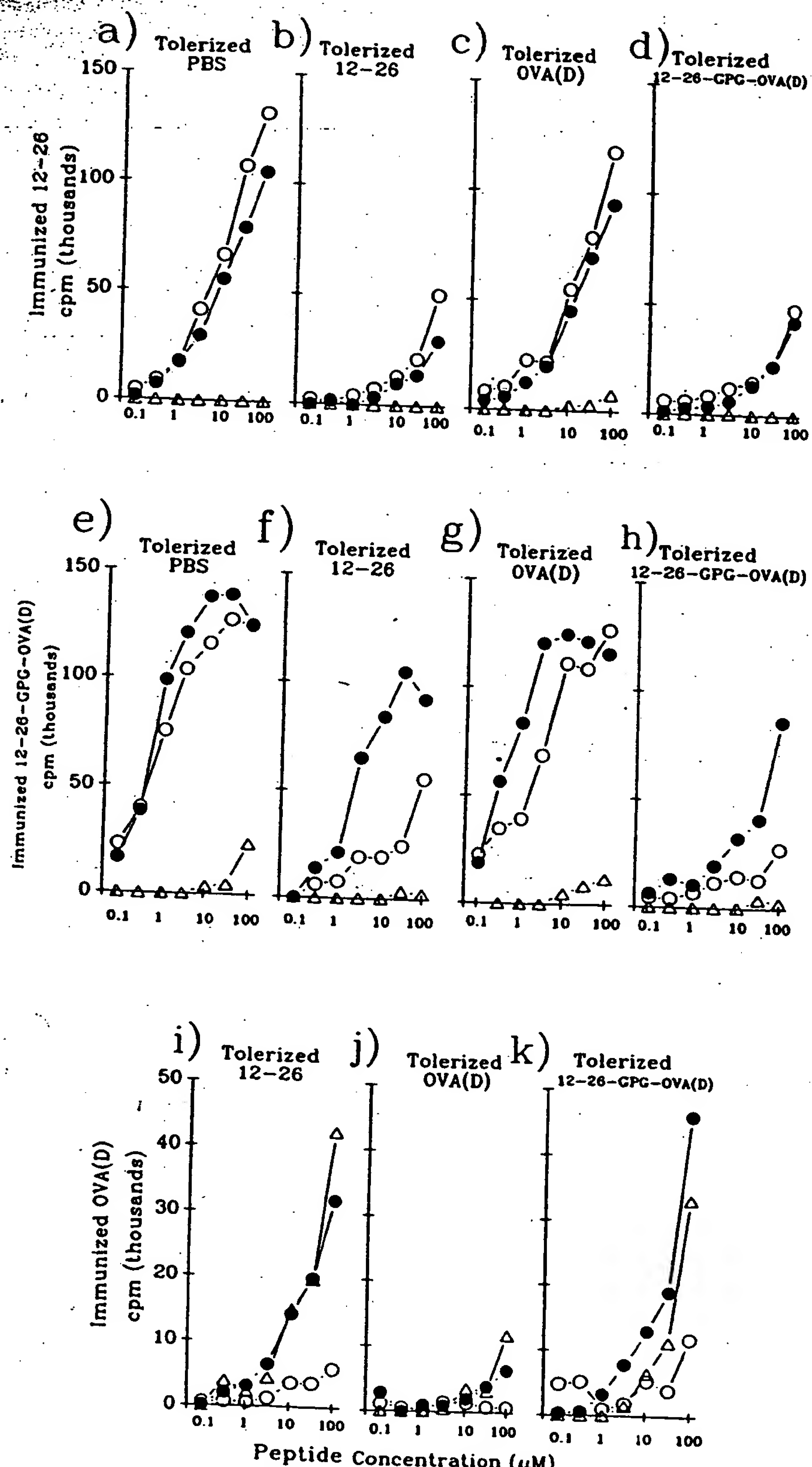


**Figure 3.** IL-2 secretion of lymphocytes from BALB/c mice. Lymphocyte response to in vitro stimulation with (●) joint peptide, (○) p12-26, (△) OVA-D. (*a*) Mice sham-tolerized and immunized with p12-26; (*b*) mice tolerized with p12-26 and immunized with p12-26; (*c*) mice tolerized with OVA-D and immunized with p12-26; (*d*) mice tolerized with joint peptide and immunized with p12-26; (*e*) mice sham-tolerized and immunized with joint peptide; (*f*) mice tolerized with p12-26 and immunized with joint peptide; (*g*) mice tolerized with OVA-D and immunized with joint peptide; (*h*) mice tolerized with joint peptide and immunized with joint peptide; (*i*) mice tolerized with p12-26 and immunized with OVA-D; (*j*) mice tolerized with OVA-D and immunized with OVA-D; (*k*) mice tolerized with joint peptide and immunized with OVA-D.

**Table 3.** Sequences of Peptides Used

| | |
|---|---|
| P12–26 | L E D A R R L K A I Y E K K K |
| P12–26$^{N14}$ | L E N A R R L K A I Y E K K K |
| OVA-D | Q A D H A A H A E I N E |
| Joint peptide | L E D A R R L K A I Y E K K K G P G Q A D H A A H A E I N E |

3j), but mice that were tolerized with p12-26 do respond (Fig. 3i). However, mice tolerized with the joint peptide and immunized with OVA-D still respond to both OVA-D and the joint peptide (Fig. 3k). Thus, the OVA-D epitope is hidden within the joint peptide during tolerization as well as immunization. It appears that tolerization to T-cell epitopes occurs when such epitopes are presented by APCs but in the absence of a "second signal."

The mode of immunization is known to influence the immune response (Warren et al. 1986). For the purposes of this study, we may ask what types of second signals promote what types of immune responses. T-cell hybridomas were prepared from BALB/c mice immunized with p1-102 in CFA s.c., CFA i.p., IFA s.c., IFA i.p., and alum i.p. They were originally screened by measuring IL-2 secretion, as tested for by the ability to sustain growth of the IL-2-dependent cell line CTLL. Previous studies (Roy et al. 1989) have shown that p12-26 is the immunodominant epitope in BALB/c mice immunized with p1-102 in CFA s.c.. As shown in Table 4, p12-26 was also immunodominant in immunized mice using the five different protocols of immunization. About 90% of all p1-102-specific hybrids respond to p12-26. In addition, about half of the non-p12-26 hybrids were found to respond to a second p1-102 peptide, p46-62.

The p46-62- and p12-26-specific hybrids were further characterized with respect to their production of IL-4, using the IL-4-dependent clone CT.4S as indicator cells. Interestingly, over 50% of the p46-62-specific hybridomas produced IL-4 upon stimulation by B cells (A20) as APCs (Table 5). In contrast, less than 5% of the p12-26-specific hybridomas produced IL-4. A representative p12-26-specific hybridoma, 1PA12-1, produced IL-2 when given p12-26 presented on either the B cell A20 or I-A$^d$-transfected L cells (Fig. 5a,b). The CTLL response was inhibitable by anti-IL-2 antibody. In contrast, there was no response when the supernatants were tested on CT.4S cells (Fig. 5c,d). Thus, T cells that recognize p46-62 often secrete IL-4, whereas those that recognize p12-26 rarely do. It is possible that this observation is due to presentation by distinct subsets of APCs that may direct responding T cells to express different functional phenotypes. We propose that APCs that can process p1-102 to give rise to p46-62 leading to IL-4 production are a subset of APCs distinct from those that process p1-102 primarily to p12-26. Evidence supportive of this possibility was obtained by comparing the ability of B cells (A20) and I-A$^d$-transfected L cells to stimulate IL-4 production from the identified hybridomas. A representative p46-62-specific hybridoma, 1SI46l-1.1, produces IL-2 when A20 cells are used as APCs, as shown by a CTLL response that is inhibitable by anti-IL-2 and not by anti-IL-4 (Fig. 6a). IL-4 production was also observed by stimulation of CT.4S, which is inhibited by anti-IL-4 and not by anti-IL-2 (Fig. 6c). The small decrease in the CT.4S response in the presence of anti-IL-2 may be due to a synergistic effect of IL-2 on IL-4 secretion on CT.4S stimulation (Hu-Li et al. 1989), as well as to the nonspecific toxicity of the antibody culture supernatant used. When I-A$^d$-transfected L cells are used to present p46-62, production of IL-2 but not IL-4 is observed (Fig. 6b,d). No hybrid tested has been able to secrete significant amounts of IL-4 when I-A$^d$-transfected L cells were used as APC. This is not due simply to a dose-response shift of the L cells as compared to A20 cells. The dose response of using L cells shifts 3-fold for the IL-2 response (Fig. 6a,b), whereas there is no IL-4 response while using L cells even at 32 $\mu$M of p46-62, 32-fold more antigen than required with A20 cells (Fig. 6c,d).

## DISCUSSION

One known mechanism of thymic tolerance depends on the deletion of thymic T cells reactive to self-peptides bound to MHC-encoded molecules of thymic APCs (Kappler et al. 1987; Kisielow et al. 1988). This

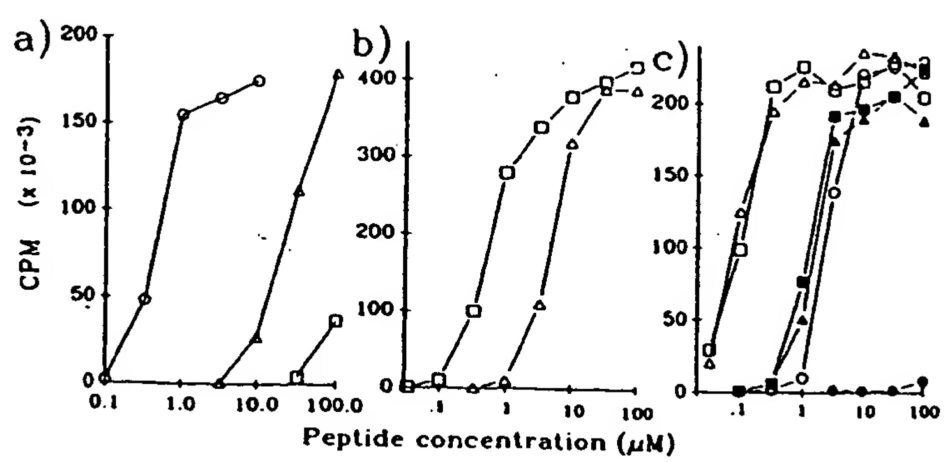


Figure 4. Hybridoma response to in vitro stimulation with peptides. Open symbols are live APCs, and filled symbols are fixed APCs. (*a*) 3D054.8 T-cell hybridoma and I-A$^d$-transfected L cells; (○) OVA, (△) joint peptide, (□) OVA-D. (*b*) 9C127 T-cell hybridoma and I-A$^d$-transfected L cells; (△) joint peptide, (□) p12-26. (*c*) 1E1 T-cell hybridoma and A20 cells; (○, ●) p1-102, (△, ▲) joint peptide, (□, ■) p12-26.

**Table 4.** Epitopes of BALB/c Hybridomas Immunized with *cI* p1–102

| Immunization protocol | Number of hybrids | | | |
|---|---|---|---|---|
| | 1–102 + | 12–26 + | 12–26⁻ tested with peptides | 46–62 + |
| Alum i.p. | 436 | 389 | 27 | 12 |
| CFA i.p. | 252 | 216 | 19 | 9 |
| CFA s.c. | 134 | 121 | 7 | 2 |
| IFA i.p. | 413 | 379 | 27 | 12 |
| IFA s.c. | 199 | 179 | 17 | 9 |
| Total | 1434 | 1284 | 97 | 44 |

mechanism can only delete those T cells that react to self-proteins that are expressed or travel to the fetal thymus. Expression in the fetal thymus of every self-T-cell epitope found in every protein in the organism would be extremely difficult. Even if tolerance is needed only for those proteins that are accessible to the immune system, and assuming efficient transport of all proteins to the fetal thymus, many proteins are only expressed after birth or later. T cells reactive to proteins expressed late in development would be able to leave the thymus and initiate autoimmune disease later in life. The fact that this does not occur argues for some sort of peripheral, continuing tolerance mechanism. We have found evidence for such a mechanism in that, by exposing a mouse to a T-cell epitope i.v. in saline or i.p. in IFA, the mouse later has a drastically diminished T-cell response to the same epitope when administered later using CFA as the adjuvant. This reduction or tolerance is epitope-specific, lasts for at least 6 weeks, and is not due to the induction of suppressor T cells. It also completely inhibits the antibody response to the epitope, presumably by blocking T-cell help.

We propose that the immune system in its resting state, i.e., unstimulated by an infectious agent or gross tissue damage, is normally in a tolerogenic state. All T cells that see antigen in that state are turned off either by clonal deletion or by clonal anergy. Only when the immune system is turned on by frank infection, by such agents as the bacterial antigens and mitogens found in the mycobacteria, or by the presence of soluble cytokines, are T cells able to respond by proliferating, secreting IL-2, and helping B cells. Furthermore, all of the T-cell epitopes we have tested that are capable of eliciting an immune response are just those epitopes that can also serve to induce tolerance. Thus, it would appear that epitopes processed and presented in the absence of a second signal lead to tolerance. The observations presented here readily explain the classic low zone tolerance to soluble proteins.

**Table 5.** IL-4-producing BALB/c Hybridomas from Mice Immunized with *cI* p1–102

| Immunization protocol | Number of IL-4-producing clones/total clones | |
|---|---|---|
| | p12–26 | p46–62 |
| Alum i.p. | 0/11 | 4/7 |
| CFA i.p. | 0/20 | 2/4 |
| CFA s.c. | 1/16 | not recovered |
| IFA i.p. | 2/30 | 4/11 |
| IFA s.c. | 1/15 | 5/5 |
| Total | 4/92 (4.3%) | 15/27 (55.6%) |

Results from the present study clearly demonstrate a regulatory effect of competing epitopes present within the same peptide upon the immune response. Thus, a majority of the T-cell response to the joint peptide is

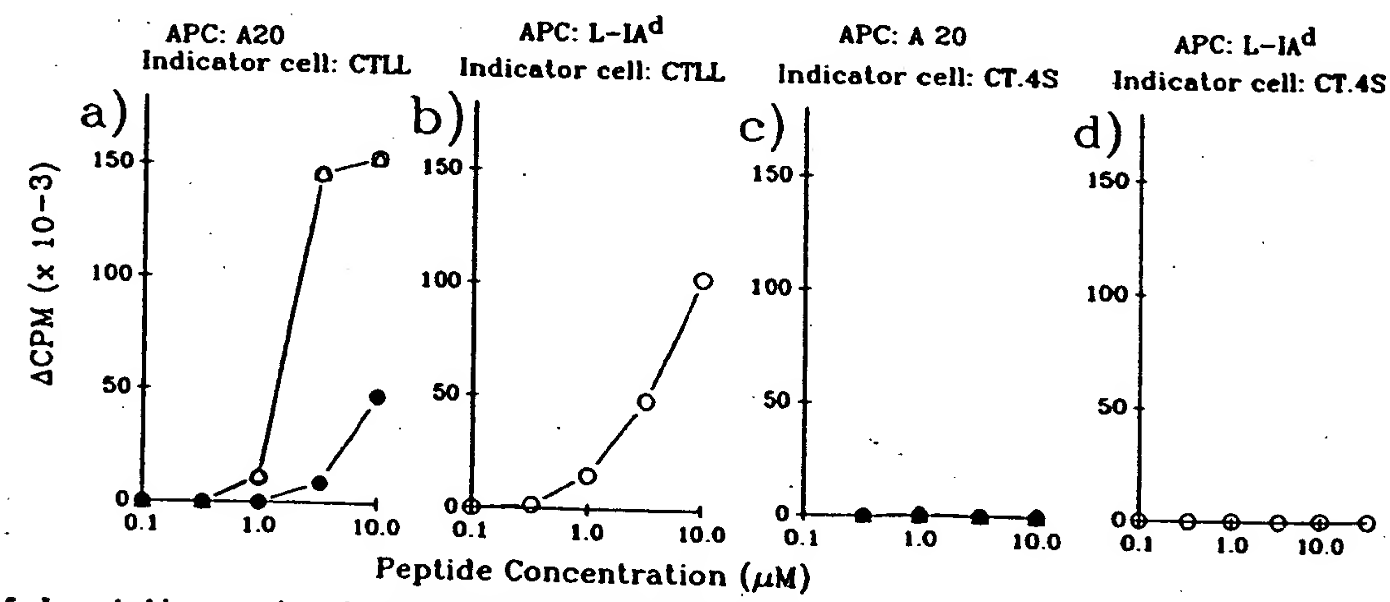


**Figure 5.** Lymphokine secretion of 1PA12-1 cells. Hybridoma response to in vitro stimulation with p12-26. Added to the indicator cells was (O) nothing, (●) anti-IL-2, (Δ) anti-IL-4. (*a*) IL-2 response with A20 cells; (*b*) IL-2 response with transfected L cells; (*c*) IL-4 response with A20 cells; (*d*) IL-4 response with transfected L cells.

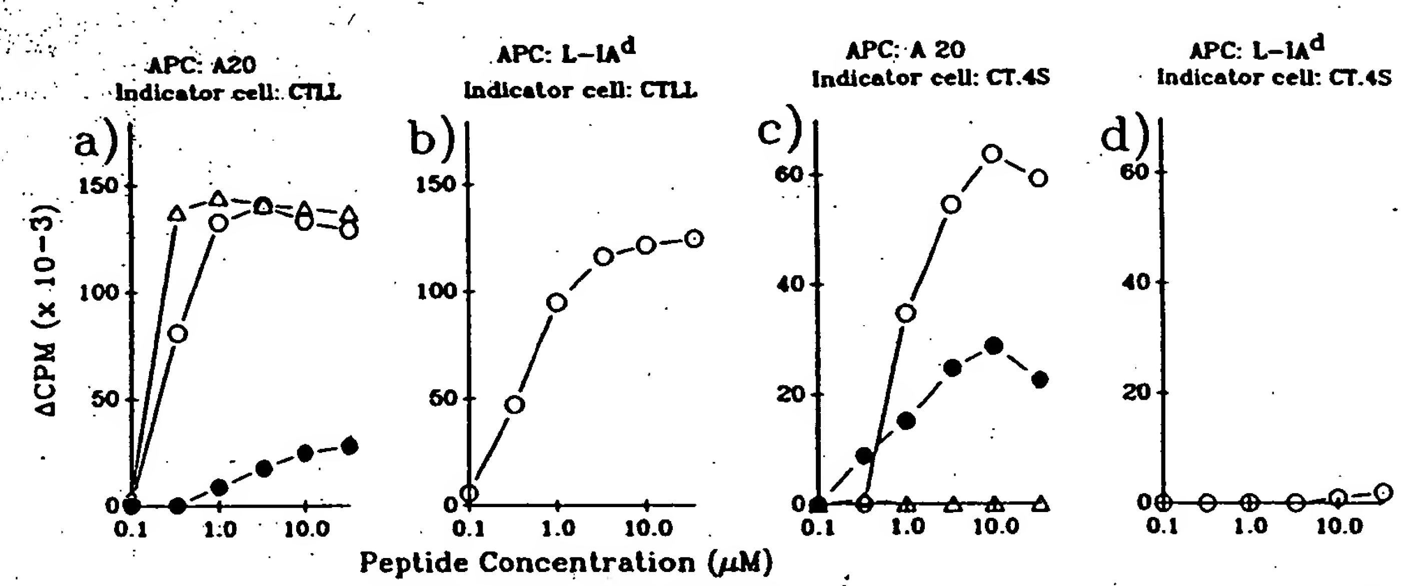


**Figure 6.** Lymphokine secretion of 1S146-1.1 cells. Hybridoma response to in vitro stimulation with p46-62. Added to the indicator cells was (O) nothing, (●) anti-IL-2, (Δ) anti-IL-4. (*a*) IL-2 response with A20 cells; (*b*) IL-2 response with transfected L cells; (*c*) IL-4 response with A20 cells; (*d*) IL-4 response with transfected L cells.

directed toward the more immunogenic epitope (12-26), leading to an apparent suppression of the response to the less immunogenic epitope (OVA-D). It is also clear that competition occurs between T-cell epitopes during the induction of tolerance. Again, the more immunogenic epitope (12-26) is tolerized, and the less immunogenic epitope (OVA-D) is ignored. The T-cell response to the less immunogenic epitope can be elicited readily in mice tolerized with the joint peptide. Therefore, the processing mechanisms of APCs inducing immunity and those inducing tolerance seem to be the same, and the less immunogenic epitopes, such as OVA-D within the joint peptide 12-26-GPG-OVA-D, are hidden for both immunization and tolerization.

Many different cell types such as B cells, macrophages, and dendritic cells can serve in vitro as APCs (Chesnut and Grey 1981; Ziegler and Unanue 1981; Sunshine et al. 1983). The involvement of the different APCs used in an immune response in vivo are unknown. However, activation of T cells by the different APCs may vary because of differences in their endocytic and processing properties (Guidos et al. 1984). Thus, the immune response may be modulated by the involvement of different types of APCs during the activation of T cells. In addition, the type of adjuvant and the route of immunization may have selective effects on the APCs used in the immune response. In our study, we observed no significant difference in the T-cell response induced in BALB/c mice immunized with λ repressor p1-102 using different combinations of adjuvants and routes of immunization. However, over 50% of T-cell hybridomas specific for p46-62 secreted IL-4, whereas less than 5% of hybridomas specific for p12-26 did. The fact that all IL-4-producing hybrids also secrete IL-2 may be attributable to the fusion partner, BW5147, which has been shown to be capable of IL-2 production (Hagiwara et al. 1988) or, more likely, to the fact that the hybrids were initially screened for their abilities to secrete IL-2. We propose the existence of at least two different types of APCs that have different antigen-processing mechanisms to explain these results. One type of APC processes p1-102 in such a way that both epitopes 12-26 and 46-62 are presented, with epitope 12-26 being dominant. T cells activated by these APCs produce only IL-2, phenotypically similar to T-helper type 1 ($T_H1$) cells. The other type of APC processes p1-102 in such a way that the 46-62 epitope predominates. T cells activated by this type of APC often secrete IL-4, phenotypically similar to $T_H2$ cells. An alternate explanation is that a single type of APC may be differentially activated to alter both its processing activity, i.e., to produce and present different epitopes, and its concomitant different second signals that alter the activation of responding T cells in such a way that their lymphokine production and thus their effect on the qualitative nature of the antibody response is altered. These possibilities are currently being explored.

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Immunology

# Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen

(orally induced immunologic unresponsiveness/autoimmunity)

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**ABSTRACT** Although oral administration of protein antigens may lead to specific immunologic unresponsiveness, this method of immunoregulation has not been applied to models of autoimmune disease. Type II collagen-induced arthritis is an animal model of polyarthritis induced in susceptible mice and rats by immunization with type II collagen, a major component of cartilage. Intragastric administration of soluble type II collagen, prior to immunization with type II collagen in adjuvant, suppresses the incidence of collagen-induced arthritis. Administration of denatured type II collagen has no observable effect on the incidence or severity of the disease. The overall magnitude of the antibody response is not significantly reduced in collagen-fed mice as compared to controls. While the isotype distribution of the anti-collagen antibodies is similar in the two groups, there is a tendency toward reduced IgG2 responses in the collagen-fed mice.

Type II collagen-induced arthritis (CIA) is an animal model of polyarthritis induced in susceptible mice and rats by immunization with type II collagen (1, 2). Type II collagen is the major matrix protein of hyaline cartilage. The similarity of the histopathologic changes observed in CIA to those seen in human rheumatoid arthritis has centered interest on the contribution of collagen autoimmunity to the pathogenesis of the human disease. Although humoral and cellular immunity to type II collagen have been shown in CIA, the precise contribution of each to the development of disease has not been established. While T cells have been shown to recognize undenatured and denatured type II collagen (3), the humoral response is restricted to the undenatured, nonrepeating helical antigenic determinants of the collagen molecule (3, 4). Development of disease after immunization with type II collagen in mice is restricted by the major histocompatability type (5). Although many mouse strains produce a vigorous humoral immune response to type II collagen, only mice of the H-$2^q$ haplotype develop arthritis. The induction of acute manifestations of CIA by the transfer of anti-type II collagen antibodies (hereafter referred to as anti-collagen antibodies) from arthritic to normal mice emphasizes the critical role of anti-collagen antibodies in the pathogenesis of CIA (6).

Several attempts to modulate the disease have led to antigen-specific suppression of collagen immunity and decreased incidence of arthritis. Induction of arthritis is suppressed by prior i.v. injection of type II collagen-coupled spleen cells (7). In rats, spleen cells from donors receiving type II collagen-coupled rat erythrocytes transfer antigen-specific suppression of CIA (8). Intravenous administration of soluble type II collagen suppresses induction of arthritis in rats and mice when given before primary immunization (9–11) or during the afferent phase of disease induction, 7–10 days after primary immunization (12).

Oral presentation of antigen is the earliest recorded method for experimentally inducing specific antigenic unresponsiveness (13, 14). This route of antigen administration can lead to immunity or tolerance, depending on the dose, number of feedings, and the form of antigen used (15). T-dependent, but not T-independent, antigens can lead to the induction of oral tolerance (16), and the immune response to collagen is T dependent (17, 18). The studies reported here show that intragastric administration of soluble type II collagen suppresses the induction of CIA in mice. It is, therefore, possible to suppress an experimental autoimmune disease by orally induced unresponsiveness.

## MATERIALS AND METHODS

**Antigens and Immunizations.** DBA/1 Lac J male mice were purchased from The Jackson Laboratories and immunized at age 8–14 weeks. Type II collagen was solubilized from fetal bovine articular cartilage by limited proteolysis with pepsin, essentially according to the technique of Trentham *et al.* (1). Collagen purity was assessed by analysis of amino acids by Genetic Design (Watertown, MA) and by $NaDodSO_4$/PAGE (19). Type II collagen was dissolved in 0.01 M acetic acid at 4°C prior to use. Denatured type II collagen was prepared by incubation at 56°C for 45 min. Intragastric feedings (0.5 ml) were administered with a ball-tipped feeding needle. Control animals were fed 0.01 M acetic acid (0.5 ml). Mice were immunized parenterally by intradermal injection of 300 $\mu$g of type II collagen emulsified in Freund's adjuvant containing heat-killed mycobacteria at 4 mg/ml (strains C, DT, and PN; Ministry of Agriculture, Fisheries and Food, Weybridge Surrey, England). Mice were boosted with 100 $\mu$g of type II collagen i.p. on day 21.

**Assessment of Arthritis.** Mice were observed two or three times each week for presence of distal joint swelling and erythema. Swelling was quantitated by measuring thickness of foot and width of ankle with a constant tension caliper (Dyer, Lancaster, PA). A mouse was considered arthritic when swelling and erythema were observed on consecutive measurement dates in at least one paw. In addition, clinical severity of arthritis was assessed by creation of an arthritic index. Each limb was subjectively graded on a scale of 0–3 (0, absence of arthritis; 1, mild swelling and erythema; 2, swelling and erythema of both tarsus and ankle; 3, ankylosis and bony deformity). A maximum arthritic index (MAI) was obtained for each mouse by summing the greatest score recorded for each limb (0, no disease; 12, highest possible

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Abbreviations: CIA, collagen-induced arthritis: MAI, maximum arthritic index.
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score) (9, 20). The MAI for each group was calculated according to the formula:

$$\frac{\text{mean MAI} \times \text{number of arthritic mice}}{\text{number of mice in the group}}$$

**Measurement of Anti-Collagen.** Antibodies to type II collagen in immune sera were measured by ELISA (21). To obtain $\mu$g/ml values of anti-collagen antibodies of each isotype, a mouse immunoglobulin reference serum (Miles Scientific, Naperville, IL) containing known amounts of each isotype was used as a standard (22). A mouse anti-collagen standard immunoglobulin preparation was purified from the sera of arthritic mice on a type II collagen-Sepharose column (23). Immulon 2 plates (Dynatech, Alexandria, VA) coated with rabbit anti-mouse immunoglobulin at 100 $\mu$g/ml in 0.016 M boric acid/0.15 M NaCl, pH 8, were blocked with the same buffer containing 2% (vol/vol) horse serum. The plates were washed and incubated with serial dilutions of affinity purified mouse anti-collagen and reference serum. The assay was developed by the addition of peroxidase-conjugated rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Miles Scientific), and the substrate ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Zymed Laboratories, South San Francisco, CA]. The cross reactivity of these sera was tested with purified mouse myeloma subclass proteins (Litton Bionetics, Organon Teknica, Charleston, SC) and found to be <10%. Absorbances at 405 nm were determined with a vertical beam spectrophotometer (Artek Systems, Farmingdale, NY). The absorbance values obtained were used to construct a standard curve for each isotype using a computer program that does a least squares fit correlating absorbance with concentration. Curves were generated through a third-order equation in which absorbance is the independent variable and, after subtracting background, the intercept is assumed to be zero. The $\mu$g/ml values obtained for anti-collagen antibodies of each isotype in the affinity-purified mouse anti-collagen standard were then used to create standard curves with type II collagen-coated plates. Anti-collagen antibodies of each isotype in immune sera were quantitated by titration on type II collagen-coated plates developed with the rabbit anti-mouse isotyping reagents described above.

Anti-collagen antibodies in immune sera were also measured by solid-phase RIA (24). Polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were coated with type II collagen at 100 $\mu$g/ml, blocked with 2% (vol/vol) horse serum, and incubated with dilutions of immune sera. The plates were developed with $^{125}$I-labeled rabbit anti-mouse Fab. Individual wells were cut out and counted in a $\gamma$ counter. Affinity-purified mouse anti-collagen antibodies were used as a standard.

**Statistical Analysis.** Comparisons of means were performed by Student's *t* test. Arthritis incidences were compared with the Fisher exact test. *P* values given are for two tailed tests.

## RESULTS

**Resistance to Arthritis Induction After Feeding of Soluble Type II Collagen.** Soluble type II collagen was administered intragastrically to CIA-susceptible DBA/1 Lac J mice prior to intradermal immunization with type II collagen in complete Freund's adjuvant. Twelve feedings (500 $\mu$g each) of type II collagen in 0.5 ml of 0.01 M acetic acid during 6 weeks significantly reduced the incidence of arthritis (Fig. 1*A*; $P <$ 0.004 for each time point beyond day 30). For the group, the MAI on day 58 was 1.9 as compared to 3.9 in controls (Fig. 1*B*). For those mice in the type II collagen-fed group that did become arthritic the mean day of onset (day 44 ± 4) was not significantly delayed compared to controls (day 41 ± 2). The clinical severity of the arthritis in these animals also did not differ significantly from that in the arthritic controls (mean MAI ± SEM on day 58 of type II collagen fed animals, 5.6 ± 0.76; mean MAI controls ± SEM, 4.8 ± 0.36; *P*, 0.34).

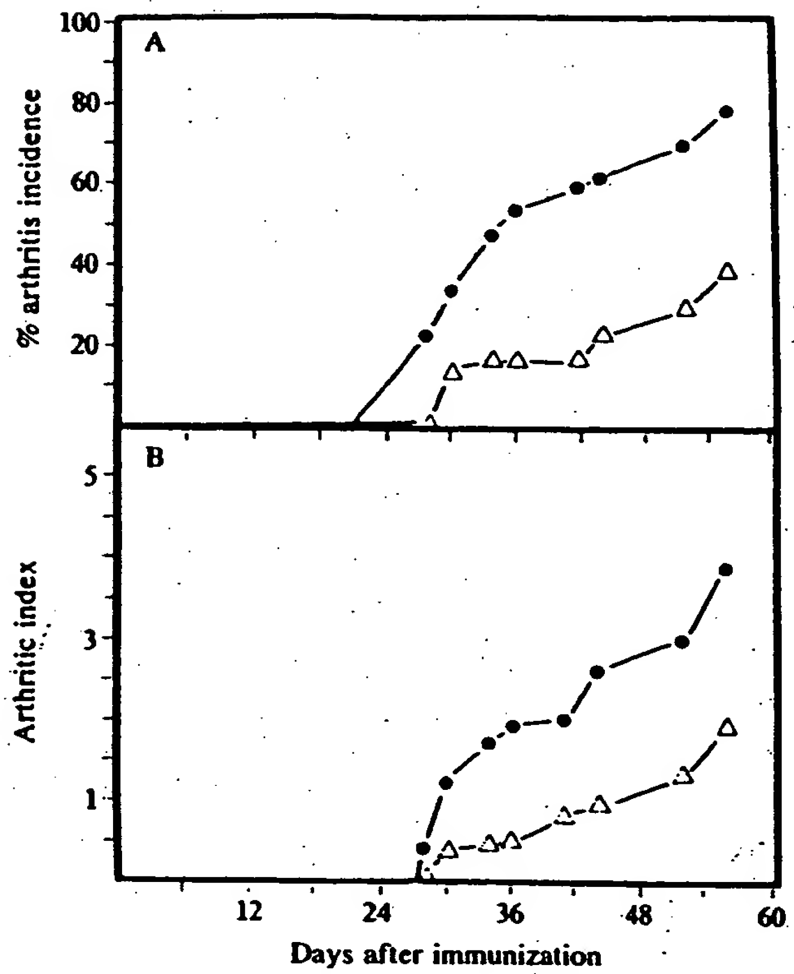


FIG. 1. (*A*) Incidence of arthritis after intragastric administration of 500 $\mu$g of type II collagen in 0.5 ml of 0.01 M acetic acid 12 times during 6 weeks (summary of six experiments). △, type II collagen ($n$ = 32); ●, controls ($n$ = 51). (*B*) Clinical severity of arthritis. △, type II collagen ($n$ = 32); ●, controls ($n$ = 51).

When both the interval and the number of feedings were decreased, it was found that eight intragastric administrations of type II collagen given over a 2-week period were sufficient to reduce the incidence of CIA significantly (Fig. 2*A*). Administration of denatured type II collagen, however, had no effect on the incidence (Fig. 2*B*) or the severity of the arthritis (MAI for the group fed denatured type II collagen, 3.4; MAI controls, 3.3). When each intragastric administration consisted of 3 mg, rather than 0.5 mg, of type II collagen no suppression of CIA was observed (Fig. 2*C*). The clinical severity of the arthritis observed in these animals was slightly higher (MAI, 4.4) than that observed in the corresponding controls (MAI, 3.7).

**Serum Antibody Levels in Type II Collagen-Fed and Control Mice.** Table 1 shows the distribution of immunoglobulin isotypes in the anti-collagen response in sera taken during the onset of disease on day 35 after primary immunization. The results are presented separately for animals with and without arthritis. The total antibody levels given are the sums of those for the individual isotypes. None of the isotypes showed statistically significant differences between arthritic and nonarthritic mice in the control group but IgG2a and IgG2b tended to be higher in the arthritic mice. IgG2b levels in type II collagen-fed mice were significantly lower than those in arthritic control mice, while IgG2a levels were also reduced, but not significantly different from those in control mice. The IgG1, IgG3, and IgA responses were lower but did not differ significantly in fed and control mice.

Since IgG2a and IgG2b were most prominent in the anti-collagen response, it was of interest to determine the

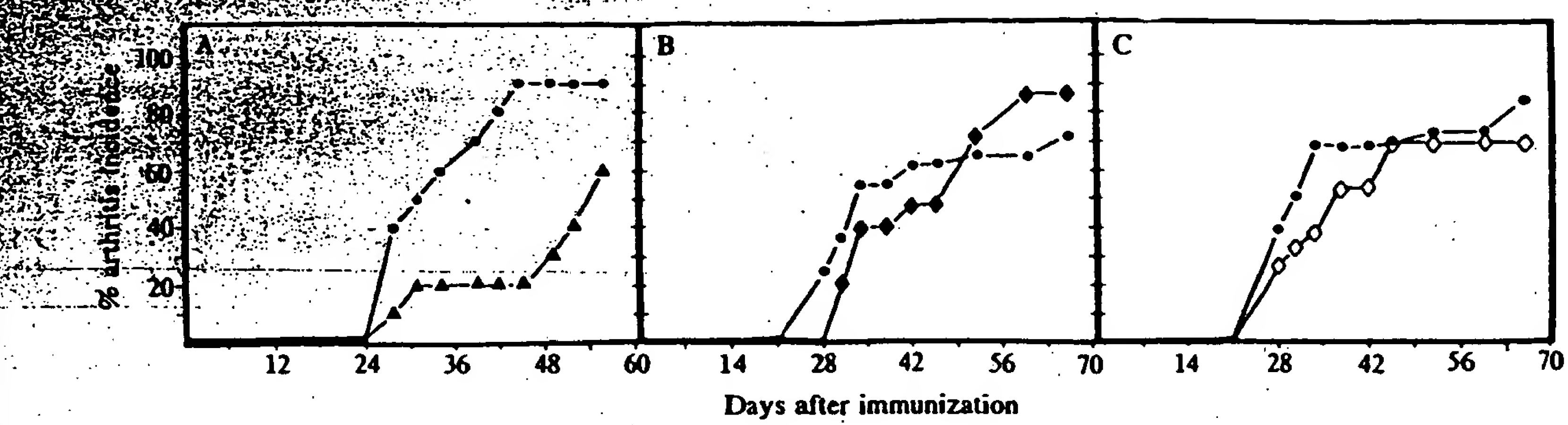


FIG. 2. Incidence of arthritis after intragastric administration of type II collagen. (*A*) type II collagen (500 μg) in 0.5 ml of 0.01 M acetic acid eight times during 2 weeks (▲, $n$ = 10). Controls 0.5 ml of 0.01 M acetic acid (●; $n$ = 10). (*B*) Denatured type II collagen (500 μg) in 0.5 ml of 0.01 M acetic acid 12 times during 6 weeks (◆, $n$ = 15). Controls (as in *A*) (●, $n$ = 28). (*C*) type II collagen (3 mg) in 1 ml of 0.01 M acetic acid 12 times during 6 weeks (○, $n$ = 19). Controls had 1 ml of acetic acid (●, $n$ = 18). Mice were immunized with type II collagen in complete Freund's adjuvant 3 days after the last dose of type II collagen.

level of these antibodies during the afferent phase of disease induction prior to onset of arthritis, as well as later in the course of the disease (Table 2). The IgG2a and IgG2b levels measured on day 20 after immunization were similar in all of the mice. In spite of the resistance to disease induction in type II collagen-fed mice, their IgG2a antibody levels were not significantly lower than those of control mice. However, the tendency to reduced IgG2a and IgG2b antibody levels observed during the onset of the disease (day 35) persisted, even in sera taken on day 60 after immunization. Measurement of total serum anti-collagen antibodies by RIA gave similar results. In a group of control ($n$ = 4) and type II collagen-fed ($n$ = 7) mice, anti-collagen levels on day 20 were 78 ± 21 and 99 ± 35 μg/ml (mean ± SEM), respectively. Sera from arthritic control mice ($n$ = 9) taken on day 60 measured 244 ± 46 μg/ml, while nonarthritic type II collagen-fed mice measured 156 ± 19 μg/ml (mean ± SEM; $n$ = 10; $P$ = 0.17).

## DISCUSSION

It has been postulated that immunologic unresponsiveness is induced after intragastric administration of antigen by the separation in the gut of tolerogenic, monomeric forms of antigen from immunogenic large molecular weight aggregates (25). After intragastric administration of soluble bovine serum albumin (25) or ovalbumin (26), only monomeric antigen could be detected in the sera of fed animals. Very small quantities of intact native proteins or small fragments bearing antigenic determinants of the native protein appear to be absorbed. In the present experiments, intragastric administration of undenatured, but not denatured, type II collagen leads to suppression of CIA. This is consistent with evidence that the immune response to type II collagen in mice is directed against the undenatured helical antigenic determinants (4, 27) and that only immunization with undenatured type II collagen readily induces arthritis (23, 27).

Eight feedings of soluble type II collagen over a 2-week period are as effective in suppressing CIA as 12 feedings over 6 weeks. In contrast, administration of large doses of type II collagen in each feeding does not result in suppression of CIA. Results from other investigators (reviewed in ref. 15) also suggest that the induction of unresponsiveness by the oral route is strikingly dose dependent. Continued feeding reduces the absorption of antigen, probably as a result of local immunity (15). It is possible that the high dose of type II collagen used here may have immunized the recipient, although no anti-collagen antibodies, even of the IgA isotype, have been detected in sera taken after feeding. It is of interest that the severity of the disease observed in the fed animals that do become arthritic does not differ from that in controls.

It has been reported that feeding of antigen subsequent to parenteral immunization either has no suppressive effect or boosts antibody production (16). Others suggest, however, that continued feeding of small doses of antigen may lead to systemic unresponsiveness in spite of initial priming (15). Repeated oral administration of ovalbumin can prevent a secondary antibody response in primed mice (28). In the present studies (data not shown) eight intragastric administrations of type II collagen given between days 10 and 29 after immunization with type II collagen in complete Freund's adjuvant did not result in decreased incidence or severity of CIA.

It has been suggested that CIA is an autoimmune disease initiated by the binding of antibody to autologous type II collagen in the joint (29). Complement is required for the development of CIA (30). Onset of disease in susceptible mouse strains is associated with a predominance of IgG2a anti-collagen whereas resistant strains mount a relatively deficient IgG2a response (22). This is in agreement with the fact that IgG2 is the most efficient member of the mouse IgG class in the fixation of complement by the classical pathway (31). The IgG2 anti-collagen response is, therefore, of par-

Table 1. Effect of type II collagen feeding on isotype distribution of serum anti-collagen on day 35 after immunization

| Pretreatment | Arthritis | No. of mice | Anti-collagen isotype, μg/ml IgG1 | IgG2a | IgG2b | IgG3 | IgA | Total immunoglobulin, μg/ml |
|---|---|---|---|---|---|---|---|---|
| Acetic acid-fed control | + | 6 | 52 ± 14 | 437 ± 108 | 107 ± 17 | 10 ± 3 | 48 ± 14 | 653 ± 132 |
| | − | 4 | 49 ± 21 | 169 ± 31 | 42 ± 11 | 6 ± 3 | 25 ± 7 | 291 ± 53 |
| | Total | 10 | 51 ± 11* | 330 ± 77† | 81 ± 15‡ | 8 ± 2§ | 39 ± 9¶ | 508 ± 98‖ |
| Collagen fed | + | 2 | 47 ± 7 | 122 ± 32 | 30 ± 9 | 4 ± 3 | 16 ± 3 | 227 ± 18 |
| | − | 6 | 34 ± 16 | 136 ± 8 | 37 ± 23 | 5 ± 4 | 16 ± 1 | 219 ± 42 |
| | Total | 8 | 44 ± 7* | 126 ± 24† | 32 ± 9‡ | 4 ± 2§ | 16 ± 2¶ | 221 ± 31‖ |

Results are mean ± SEM. +, Mice with arthritis. −, Mice without arthritis.
*$P$, 0.64; †$P$, 0.09; ‡, 0.03; §$P$, 0.19; ¶$P$, 0.12; ‖$P$, 0.06.

Table 2. IgG2a and IgG2b serum anti-collagen prior to and late in the development of CIA in type II collagen-fed and control mice

| Day | Pretreatment | Arthritis | No. of mice | Anti-collagen isotype, μg/ml IgG2a | IgG2b |
|---|---|---|---|---|---|
| 20 | Acetic acid-fed control | − | 5 | 70 ± 8* | 19 ± 6† |
| | Collagen fed | − | 8 | 98 ± 36* | 16 ± 4† |
| 60 | Acetic acid-fed control | + | 9 | 448 ± 134‡ | 32 ± 7§ |
| | Collagen fed | + | 4 | 387 ± 143 | 59 ± 17 |
| | | − | 9 | 294 ± 166‡ | 12 ± 2§ |

Results are mean ± SEM. +, Mice with arthritis. −, Mice without arthritis.
*P, 0.6; †P, 0.7; ‡P, 0.49; §P, 0.06.

ticular importance for the development of CIA. The possibility exists that resistance to induction of CIA observed after intragastric administration of type II collagen results from a decrease in the magnitude of the IgG2 response or a switch in isotype predominance. We have found that while the overall anti-collagen response is slightly, but not significantly lower, IgG2a remains the predominant isotype in both fed and control mice. In addition, although much lower in magnitude than the IgG2a response, the IgG2b anti-collagen response is significantly lower in nonarthritic type II collagen-fed mice than in arthritic control mice.

There are at least two possible mechanisms for the resistance to arthritis induction observed after feeding of soluble type II collagen. Mattingly and Waksman (32) found that feeding sheep erythrocytes for 2 weeks resulted in systemic unresponsiveness. Within 2 days after feeding, suppressor T cells appeared in the Peyer's patches and mesenteric lymph node, but were undetectable in these locations and present in the spleen and thymus after 4 days. Suppressor T cells have also been shown, by adoptive transfer, in the mesenteric lymph node and spleen of animals suppressed by oral administration of soluble proteins or haptens in several other experimental systems (33–38). In the present system collagen-specific suppressor T cells could have suppressed antibody production, particularly of the IgG2 class, or might have prevented the sensitization of T cells directly involved in the initiation and maintenance of arthritis. Alternatively, production of anti-idiotypic autoantibody has been postulated to depress specificially the IgM and IgG immune response after systemic challenge of fed animals with an immunizing dose of antigen (39, 40). Although in the present study the effect of type II collagen feeding on the magnitude of the humoral anti-collagen response is not marked, it is still possible that anti-idiotypic regulation could have suppressed a part of the response critical for the induction of CIA by virtue of its specificity.

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# SUPPRESSION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY ORAL ADMINISTRATION OF MYELIN BASIC PROTEIN AND ITS FRAGMENTS[1]

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**We report that experimental autoimmune encephalomyelitis, a T cell-mediated autoimmune disease studied as a model for multiple sclerosis, can be suppressed in Lewis rats by the oral administration of myelin basic protein (MBP). Both the clinical and histopathologic manifestations of the disease were suppressed in a dose-dependent manner. In addition, proliferative responses to MBP and, to a lesser extent, serum levels of anti-MBP antibody were suppressed by feeding MBP. Suppression of clinical and histologic disease was observed whether animals were fed MBP before or after disease induction, although suppression was more complete when rats were fed before immunization. Disease was also suppressed by the oral administration of either encephalitogenic or nonencephalitogenic fragments and decapeptides of the MBP molecule, with more complete suppression observed when nonencephalitogenic fragments were fed, suggesting that suppressor determinants exist in the MBP molecule distinct from the encephalitogenic region. The oral administration of a non-disease-inducing portion of an autoantigen represents an antigen-specific method by which an experimental autoimmune disease can be immunoregulated.**

Experimental autoimmune encephalomyelitis (EAE)[3] is a T cell-mediated autoimmune disease directed against myelin basic protein (MBP) and has been studied in several mammalian species as a model for the human demyelinating disease multiple sclerosis (1). Several studies have focused on methods to suppress the development and severity of EAE. For example, it has been demonstrated that suppression of the disease is possible by non-antigen-specific methods such as treatment with cyclophosphamide (2) or injection of monoclonal antibodies directed against T cell subsets (3, 4). In addition, various antigen-specific methods have been used to suppress EAE, including systemically administered antigen or antigen conjugated to lymphoid cells (5–7).

An effective and long recognized method of inducing immunologic tolerance is the oral administration of antigen (see Reference 8 for review), which was first demonstrated by Wells for hen's egg proteins in 1911 (9). Oral induction of immunologic unresponsiveness has been demonstrated for a number of T-dependent, but not T-independent, antigens (8, 10) and we have previously investigated oral tolerance to viruses (11). Orally induced tolerance has been shown in several instances to be the result of the generation of antigen-specific suppressor T cells (11–19), although other mechanisms including antiidiotypic antibodies and immune complexes have also been implicated as being responsible for the induction of oral tolerance (20–22). In the present report, we have studied the effect of feeding MBP on the susceptibility to and severity of acute monophasic EAE in the Lewis rat. In addition, because only a small segment of the MBP molecule is known to be encephalitogenic and the remainder is unable to induce disease (23), we also examined the effect of oral administration of peptide fragments of MBP on the development of EAE. Our results show that the oral route of antigen administration suppresses not only disease but also various immune responses to MBP and that suppression of EAE is also possible by feeding nonencephalitogenic fragments of the MBP molecule.

## MATERIALS AND METHODS

*Rats.* Lewis rats were obtained from the Charles River Laboratory (Wilmington, MA). Animals were used at 6 to 8 wk of age.

*Antigens.* Guinea pig and bovine MBP were purified from brain tissue by the method of Diebler et al. (24). Fragments of guinea pig MBP were generated by limited pepsin digestion at 25°C, pH 3.0, and were separated by ion exchange chromatography as described (25). The peptides were run on phosphoric acid-urea sodium dodecyl sulfate-polyacrylamide gels (26) to verify their homogeneity. The bovine encephalitogenic decapeptide (Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-Asn) and the decapeptide S79, which differs from the encephalitogenic decapeptide by a single amino acid substitution (Gly for Asn) at the carboxyl terminus, were synthesized using an Applied Biosystems 430A peptide synthesizer.

*Induction of oral tolerance.* Rats under light ether anesthesia were fed MBP or fragments in 0.25 ml of phosphate-buffered saline (PBS) solution by using a syringe fitted with a 20-gauge ball point needle. Control animals were fed equal amounts of bovine serum albumin (BSA) or saline alone.

*Immunizations.* EAE was induced in Lewis rats by immunization with guinea pig MBP emulsified in complete Freund's adjuvant (CFA). A total of 0.4 ml of emulsion containing 50 $\mu g$ of MBP in 0.2 ml saline and 0.2 ml of CFA was injected into the hind footpads of each rat. Disease was characterized by hind limb paralysis and incontinence, usually between days 12 and 15 after immunization, and in all cases rats recovered by day 16. Clinical scoring was as follows: 1 = tail weakness; 2 = tail weakness plus hind limb weakness; 3 = hind limb paralysis plus incontinence; and 4 = Moribund.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; CFA, complete Freund's adjuvant; PBST, phosphate-buffered saline containing 0.05% Tween 80.

*Histology.* Rats were killed on day 16 after immunization and brains were removed and fixed in a solution of 3% formaldehyde, 60% ethanol, and 4% acetic acid. Slides of paraffin-embedded tissue were prepared from each individual rat and 10-μm sections were stained with hematoxylin and eosin. Numbers of perivascular inflammatory foci were quantified from coded slides by established procedure (27).

*Proliferation assay.* Ten days after immunization, some of the rats from each experimental group were killed, the popliteal lymph nodes were removed, and single cell suspensions were prepared. A volume of 0.2 ml containing $4 \times 10^5$ cells in RPMI 1640 containing 2% glutamine, 1% penicillin/streptomycin, $5 \times 10^{-5}$ M 2-mercaptoethanol, and 5% fetal calf serum was added to each microwell and MBP was added at 50 μg/ml. The cells were cultured for 72 hr, each well was pulsed with 1 μCi of tritiated thymidine, and cells were cultured for another 24 hr. Cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques. All cultures were performed in triplicate and results were expressed as stimulation indexes (experimental cpm/control cpm).

*Measurement of serum antibody.* An enzyme-linked immunosorbent assay was used to measure the level of serum anti-MBP antibody in rats. A volume of 0.1 ml of MBP solution (0.05 mg/ml in PBS) was added to each microwell and was incubated for 3 hr at 37°C. Wells were washed with PBS containing 0.05% Tween 80 (PBST) and were blocked overnight at 4°C with 5% BSA in PBS, pH 9.0. After washing with PBST, rat sera (diluted in PBS) were added and incubated for 3 hr at room temperature and, after washing with PBST, secondary antibody (peroxidase-conjugated goat anti-rat IgG) was added for 1 hr at room temperature. Substrate was added and the reaction was stopped with 0.1 M NaF. Plates were read at 450 nm on a Titertek multiscan. Absorbance at 450 nm was also determined from serum from rat immunized with only CFA and was subtracted from all values as background.

## RESULTS

*Oral tolerance of EAE is dose-dependent.* The first series of experiments investigated the effect of the number of feedings and the dose of MBP on the suppression of disease. Rats were fed various amounts of MBP either once 7 days before (day −7) the day of immunization (day 0) or three times on days −14, −7, and 0. The results (Table I) demonstrate that feeding MBP to rats suppresses EAE and that orally induced suppression is dose dependent. Multiple 500-μg feedings resulted in complete suppression of disease and were more effective than a single feeding at this dose. In addition to clinical manifestation of EAE, histologic evidence of disease in rats was examined. Rats were sacrificed 16 days after immunization and brains were removed and fixed in formalin solution. As shown in Table I, feeding rats 500 μg of MBP on days −14, −7, and 0 caused a marked decrease in the number of inflammatory lesions in the brain. A moderate decrease was found in animals fed 100 μg and no significant reduction of inflammation was found in rats fed 25 μg of MBP.

*Effect of feeding MBP before or after immunization.* A second series of experiments investigated the effect of feeding MBP before or after immunization with MBP to determine whether orally induced suppression is affected by previous exposure to antigen. For these experiments, animals were fed 500 μg of MBP three times within a narrower time frame either before (days −7, −5, and −2) or after (days +2, +5, and +7) active induction of disease (immunization with MBP). The results (Table II) show that the clinical expression of disease is suppressed whether animals were fed MBP before or after sensitization, the effect being more complete when antigen was fed before immunization. However, histologic examination revealed a marked reduction of perivascular infiltrates in rats fed MBP either before or after sensitization to MBP. Greater than 60% suppression of clinical disease was also observed when rats were fed 500 μg of MBP three times beginning on day +5 or on day +7 after immunization (data not shown).

Further experiments were conducted to determine the persistence of orally induced protection against EAE. After receiving 500 μg of MBP on days −7, −5, and −2, rats were immunized at various lengths of time after the last feeding. EAE was completely suppressed in rats at 4 wk after feeding; by 8 wk 50% of animals were again susceptible to disease (data not shown).

To determine whether suppression of EAE by feeding MBP was specific for MBP and not due to a nonspecific effect of a basic protein, rats were fed 500 μg of histone three times (days −7, −5, and −2) and immunized with MBP. Feeding histone had no effect on the incidence of EAE (data not shown).

*Effect of feeding MBP on immune responses to MBP.* The effect of oral administration of MBP on cellular and humoral immune responses to MBP was also examined. Proliferative responses to MBP were studied after feeding rats different doses of MBP and following feeding at different times with respect to immunization. Rats were killed 10 days after immunization and proliferative responses of draining lymph node cells were determined. The results (Fig. 1) demonstrate that feeding MBP before immunization caused a pronounced decrease (75 to 92% suppression) of the proliferative response to MBP. Suppression of proliferation, unlike suppression of disease, occurred at all doses and feeding regimes tested. Feeding MBP after immunization was also effective in suppressing the proliferative response to MBP (Fig. 2). Orally induced suppression of the proliferative re-

TABLE I
*Effect of feeding dose on orally induced suppression of EAE*

| MPB Feeding (μg)[a] | Clinical Disease[b] | Histologic Score[c] |
|---|---|---|
| Control | 19/22 | 9.2 ± 5.8 |
| Day −7 | | |
| 25 | 3/5 | ND |
| 100 | 2/5* | ND |
| 500 | 3/10*** | ND |
| Days −14, −7, 0 | | |
| 25 | 3/5 | 7.4 ± 5.2 |
| 100 | 2/5* | 3.2 ± 1.9 |
| 500 | 0/10*** | 0.2 ± 0.4 |

[a] Rats were fed various doses of MBP on the indicated days and immunized with 50 μg of MBP in CFA (200 μg of *M. tuberculosis*) on day 0. Shown are the number of diseased rats of the total number tested. Immunized controls were fed BSA or saline. Clinical disease in controls and in fed animals consisted of hind limb paralysis and incontinence.

[b] Groups were compared with immunized controls by $\chi^2$ analysis with one degree of freedom. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[c] Rats were killed on day 16 after immunization and brains were removed and fixed. Shown are the average number of perivascular inflammatory foci per group ± SD. ND, not determined.

TABLE II
*Effect of feeding MBP to rats before or after immunization on the development of EAE*

| MBP Feeding[a] | Clinical Disease[b] | Histologic Score[c] |
|---|---|---|
| Control | 23/26 | 21.6 ± 5.1 |
| Days | | |
| −7, −5, −2, +2, +5, +7 | 0/5*** | 0.2 ± 0.4 |
| −7, −5, −2 | 0/17*** | 0 |
| +2, +5, +7 | 4/10** | 1.4 ± 2.3 |

[a] Rats were fed 500 μg of MBP on the indicated days and immunized with 50 μg of MBP in CFA on day 0. Immunized controls were fed BSA or saline. Clinical disease in controls and fed animals consisted of hind limb paralysis and incontinence.

[b] Groups were compared with immunized controls by $\chi^2$ analysis with one degree of freedom. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[c] See Table I.

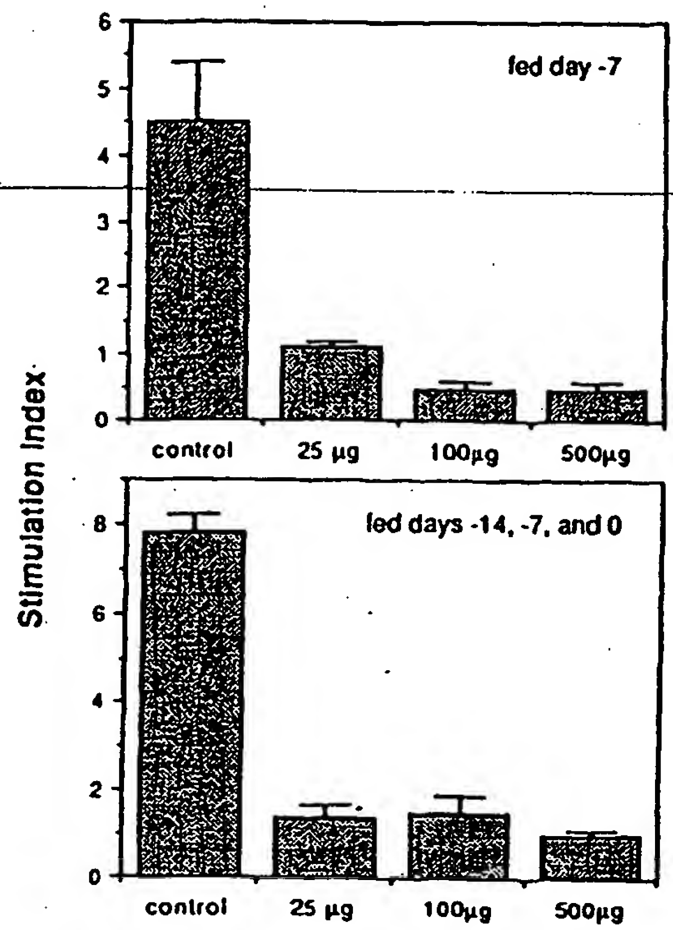


*Figure 1.* Effect of dose and number of feedings of MBP on proliferative response to MBP. Animals were fed various doses on the indicated days and then immunized with 50 µg of MBP in CFA on day 0. Proliferative response of popliteal lymph nodes was determined 10 days later.

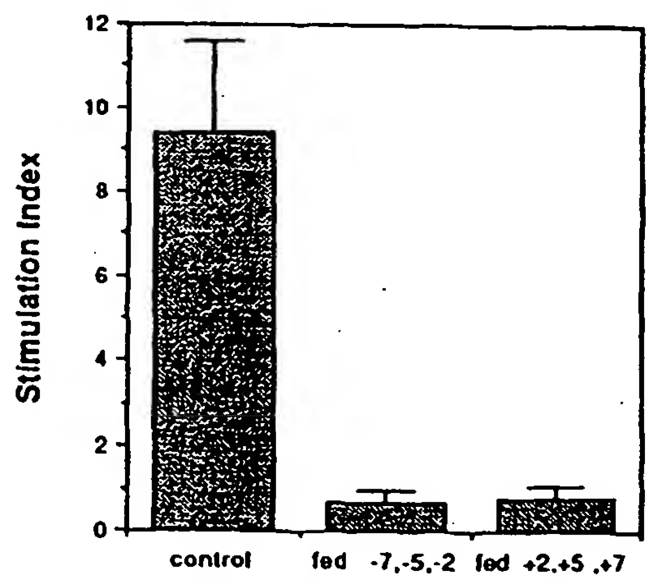


*Figure 2.* Effect of feeding MBP before or after immunization on proliferative response to MBP. Animals were fed 500 µg of MBP on the indicated days and immunized with 50 µg of MBP in CFA on day 0. Proliferative response of popliteal lymph nodes was determined on day 10 after immunization.

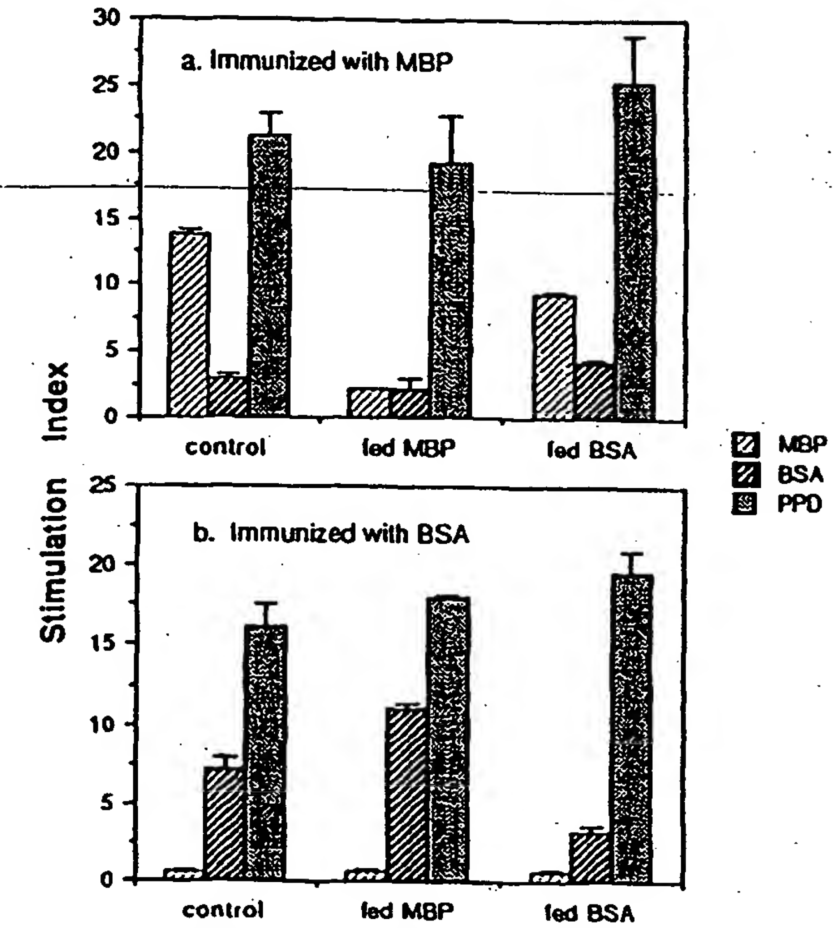


*Figure 3.* Antigen specificity of orally induced suppression of the proliferative response. Animals were fed 500 µg of MBP or BSA on days −7, −5, and −2 and immunized with 100 µg of MBP or BSA in CFA on day 0. Nine days after immunization, lymph nodes were removed and proliferative response to MBP, BSA, and a purified protein derivative of *Mycobacterium tuberculosis* (PPD) (all at 50 µg/ml) was determined.

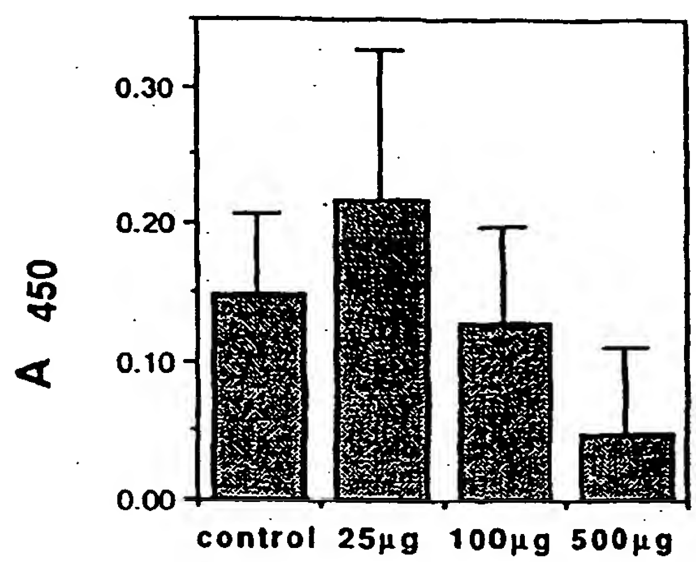


*Figure 4.* Effect of feeding MBP on the production of serum anti-MBP antibody. Animals were fed the indicated amounts of MBP on days −14, −7, and 0 and immunized with 50 µg of MBP in CFA on day 0. Rats were bled 16 days after immunization and serum antibody levels were determined by enzyme-linked immunosorbent assay. Shown are the relative absorbances at 450 nm of serum diluted 1/15,625 with respect to serum obtained from control animals immunized with CFA alone.

sponse to MBP is antigen specific, as shown in Figure 3. Specifically, the proliferative response to a purified protein derivative of *Mycobacterium tuberculosis*, which occurs as a result of immunization with CFA, is not suppressed by feeding MBP. Feeding an irrelevant antigen such as BSA also does not affect the proliferative response to this protein derivative and only slightly suppresses the proliferative response to MBP. Finally, feeding MBP does not affect the proliferative response to BSA in animals immunized to BSA.

The effect of feeding MBP on the production of antibody to MBP was also examined. Rats fed MBP were immunized with MBP in CFA and bled 16 days after immunization. Levels of anti-MBP antibody in the serum were measured by enzyme-linked immunosorbent assay. When animals were fed at weekly intervals (days −14, −7, and 0), suppression of anti-MBP antibody response was only observed when rats were fed the highest (500 µg) dose tested (66% suppression, Fig. 4). Unlike the suppression of proliferative response, suppression of antibody production was not observed when rats were fed lower doses of MBP. When rats were fed antigen either before or after immunization under the other feeding schedule (days −7, −5, and −2 or +2, +5, and +7) the antibody response was not significantly affected, with only 15% suppression observed for both pre- and postfed rats (data not shown).

*Effect of feeding MBP fragments on EAE.* It has been

reported that the encephalitogenic region of MBP for Lewis rats is a specific decapeptide sequence located at residues 75 to 84 of either guinea pig or bovine MBP, which by itself can induce EAE when injected into animals with CFA, whereas other regions of the molecule are nonencephalitogenic (23). Furthermore, for some antigens, including MBP, it has been reported that distinct suppressor determinants exist at sites different from immunogenic determinants (33, 34). We therefore investigated whether either encephalitogenic or nonencephalitogenic regions of MBP could by themselves prevent EAE via oral administration. Fragments of guinea pig MBP were generated by limited pepsin digestion and separated by column chromatography. The three different fragments generated by this procedure were fed separately to rats, and then animals were immunized with whole guinea pig MBP. It was found that both the disease-inducing (fragment 44–89) and nonencephalitogenic (fragments 1–37 and 90–170) fragments suppressed EAE when fed to rats, the nonencephalitogenic fragments being more effective in suppressing the disease than the encephalitogenic fragment (Table III). Furthermore, oral administration of both the encephalitogenic fragment (fragment 44–89) and the nonencephalitogenic fragment (fragment 1–37) suppressed subsequent proliferative response to whole MBP (Table III).

In terms of bovine MBP, it has been reported that a decapeptide, termed S79, which differs from the 75–84 encephalitogenic decapeptide by a single amino acid substitution (Gly for Asn) at the carboxyl terminus, suppresses EAE when injected into Lewis rats with CFA (28). We thus synthesized both the bovine encephalitogenic decapeptide and S79 decapeptide and fed them to animals before immunization with whole guinea pig MBP. The S79 decapeptide suppressed EAE and appears to be more suppressive of disease than the encephalitogenic decapeptide itself (Table III). Whole bovine MBP, which is not encephalitogenic in rats at doses encephalitogenic for guinea pig MBP (29), completely suppressed disease when fed to animals at the same dose as guinea pig MBP before immunization with guinea pig MBP (Table III).

## DISCUSSION

Our results demonstrate, first, that oral administration of MBP is an effective means by which acute monophasic EAE in rats can be suppressed in an antigen-specific manner. Orally induced suppression is dose-dependent, with both clinical and histologic symptoms of disease being dramatically reduced. Second, feeding MBP to rats profoundly suppresses T cell proliferative response to MBP and, to a lesser extent, anti-MBP antibody production, which is consistent with orally induced suppression of immune responses for other antigens (8). Third, nonencephalitogenic fragments of MBP (peptides 1–37 and 90–170) are as effective in orally suppressing EAE as the intact protein. Thus, oral suppression of disease does not require the disease-inducing antigenic determinant of MBP.

The oral administration of MBP was most effective in suppressing EAE when administered before immunization, suggesting that the afferent limb of disease induction was affected most by feeding. Nonetheless, feeding after the induction of disease (starting as late as day +7) was also effective in at least partially suppressing disease, which indicates that later stages of the immune response following disease induction may also be susceptible to oral suppression. Studies are currently in progress to determine the effectiveness of orally administered MBP in suppressing adoptively transferred and chronic relapsing EAE.

Although feeding MBP after EAE induction did not completely suppress disease (60% suppression vs 100% suppression if animals were fed before immunization), it was as effective as feeding before immunization in suppressing the proliferative response to MBP (90% suppression). Different doses of orally administered MBP were required to suppress disease, proliferative responses, and antibody production, and, in the case of the antibody response, the interval of feedings influenced the suppressive effect. This suggests that different mechanisms may be involved in the induction and maintenance of oral tolerance for cell-mediated and humoral responses (30, 31) and for disease protection.

We have shown that orally administered fragments of MBP that do not contain the encephalitogenic sequence prevent disease and suppress proliferative responses to MBP, suggesting that suppressor determinants exist in the MBP molecule distinct from the encephalitogenic region and that these determinants can induce tolerance when dissociated from the disease-inducing determinant. Furthermore, our data suggest that nonencephalitogenic determinants may be more potent suppressors of disease than encephalitogenic determinants. Suppressor determinants that are distinct from antigenic determinants have been described in

TABLE III

*Effect of feeding encephalitogenic and nonencephalitogenic fragments and peptides on the development of EAE*

| Feeding (μg)[a] | Incidence of EAE[b] | Proliferative Response[c] |
|---|---|---|
| Controls | 19/25 | 7.0 |
| Guinea pig MBP | | |
| Fragment 1–37 (109) | 0/9*** | 1.3 |
| Fragment 44–89 (135) | 3/11** | 2.5 |
| Fragment 90–170 (235) | 0/4** | ND |
| Bovine MBP | | |
| Whole (500) | 0/10*** | ND |
| Decapeptide S79 (30) | 1/8*** | ND |
| Encephalitogenic decapeptide (30) | 4/8 | ND |

[a] Lewis rats were fed the indicated amounts of peptides or proteins (equimolar to 500 μg of whole guinea pig MBP) on days −7, −5, and −2 and immunized on day 0 with 50 μg of guinea pig MBP with CFA. Shown are the number of diseased rats of the total number tested. Clinical disease in controls and fed animals consisted of hind limb paralysis and incontinence.

[b] Groups were compared with immunized controls by $\chi^2$ analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[c] Proliferative response of lymph node cells to whole MBP is expressed as stimulation index (experimental cpm/control cpm). ND, not determined.

hen's egg white lysozyme and appear in the amino terminus of the molecule (32, 33). Using the same EAE system we have studied (guinea pig MBP in Lewis rats), Chou et al. (34) reported that the encephalitogenic region of the MBP molecule was necessary for suppression of EAE when given with incomplete Freund's adjuvant (IFA). Driscoll et al. (35) reported similar results with the guinea pig. Swanborg (36), on the other hand, using guinea pig MBP in guinea pigs reported that an inhibitory determinant was present in fragment 43–89, which is distant from the encephalitogenic site (residues 114 to 121) and which suppressed disease when administered with IFA. The disparity between our results and those of Chou et al. (34) may relate to the differences in routes of administration of antigen used to induce suppression. The oral route may provide unique conditions that maximize the induction of suppression. For example, gut processing of antigen may modify and "biologically filter" antigen (37) in a manner that cannot be reproduced by other means, such as immunization with IFA or direct i.v. injection. In addition, the gut may provide specialized antigen-presenting cells, either in the intestinal epithelium (38) or Peyer's patch (39), which have been reported to be involved in activating suppressor inducer T cells. Studies are currently in progress to define the minimal amino acid sequences on the nonencephalitogenic determinants required to induce oral tolerance to EAE.

Orally induced tolerance is a normal immune response that is considered to function in the prevention of allergic and autoimmune reactions to food antigens (8). Although the oral administration of antigen has been widely studied as a means of suppressing the immune response for a number of different cellular (14, 15), protein (12, 13, 16, 17), and nonprotein (e.g., contact-sensitizing) antigens (11, 19), it has not been applied in the suppression of an autoimmune disease to a defined antigen until recently. We are aware of one published report in which the oral administration of type II collagen was used to suppress collagen-induced arthritis in mice (40) and of a preliminary report of EAE suppression by oral administration of MBP, in which 20 mg of MBP was fed and 58% of animals were protected from clinical disease (41). In the study of arthritis, collagen-induced arthritis was suppressed by feeding undenatured but not denatured type II collagen. There was a slight decrease in IgG2b anticollagen antibodies reported; T cell responses were not determined.

The specific immune mechanism by which oral administration of MBP or its fragments suppresses EAE is at the present time undefined. Adoptive transfer studies with animals fed other antigens (11–14, 17–19) have often shown that antigen-specific suppressor T cells are generated by feeding and are involved in actively suppressing the immune response. Other mechanisms, such as the production of soluble factors in the serum (20) and the formation of antigen-antibody complexes (21), have also been proposed and may represent additional or alternative mechanisms. Suppressor T cells have been shown to play a role in the modulation of EAE, being involved in the recovery from disease in rats (42, 43) and in the natural unresponsiveness to the disease in certain strains of mice (44). It is therefore possible that MBP-specific suppressor T cells are induced by orally administered MBP. Studies are presently in progress to determine the role of suppressor T cells in generating oral suppression of EAE via adoptive transfer of orally induced suppression from fed donors to naive recipients.

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